UNIVERSITAT POLITÈCNICA DE CATALUNYA

Laboratori de Toxicologia Ambiental

Intexter

Centre de Recerca i Innovació en Toxicologia (CRIT)

Comparative study of in vitro cell based assays versus in vivo toxicity tests to monitor environmental hazard of pesticides

Memòria presentada per a optar al grau de Doctor per
la UPC dintre del programa: Enginyeria de Projectes:
Medi Ambient, Qualitat, Seguretat i Comunicació.

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Centre de suport a la Innovació Tecnològica. Xarxa IT del CIDEM

Terrassa, 2006

El present treball d'investigació recollit en aquesta memòria ha estat realitzat per Mª Pilar Alañón Ribas, per a optar al Grau de Doctor per la Universitat Politècnica de Catalunya (UPC) dintre del programa: Enginyeria de Projectes: Medi Ambient, Qualitat, Seguretat i Comunicació.

Aquesta tesi doctoral ha estat realitzada al Laboratori de Toxicologia Ambiental (Intexter) de la Universitat Politècnica de Catalunya sota la direcció de la Dra. Mª Carme Riva Juan. Cal remarcar que una petita part del treball de recerca s'ha portat a terme a la Facultat de Veterinària de la Universitat de Milan, Itàlia, vinculada a un projecte conjunt de recerca de les dues entitats.

Així mateix s'ha de fer constar que Mª Pilar Alañón Ribas, ha gaudit d'una beca atorgada pel propi laboratori durant tot el temps necessari per tal de portar a terme la present tesi doctoral.

Terrassa, Juliol 2006

Dra. Mª Carme Riva Juan
Directora de la Tesi doctoral

Als meus pares i la meva àvia

A les meves germanes

Al meu estimat Israel

AGRAÏMENTS

En primer lloc voldria agrair especialment a la Dra. Mª Carmen Riva, directora del Laboratori de Toxicologia Ambiental, per haver-me acceptat sota la seva direcció al Laboratori de Toxicologia Ambiental de la UPC i donar-me la oportunitat de desenvolupar aquesta tesi. Gràcies sobre tot pel seu interès, ajuda i consells i pel seguiment d'aquest treball durant aquest anys.

Agraeixo també el suport econòmic dels projectes REN2003-06917-C02-02 i CGL2004-03514 així com també l'ajut concedit pel MEC, SB2003-0268 que ha permès la col·laboració amb la Facultat de Veterinària de la Universitat de Milan, Itàlia.

A la Dra. Simona Stacchezzini per ensenyar-me a treballar amb cultius primaris de granulosa bovina, pels seus útils consells sobre la ciència i sobre la vida. Gràcies també a la Dra. Francesca Caloni per la seva participació en aquest treball.

També voldria agrair a l'escorxador de Sabadell per la seva col·laboració en la presa de mostres d'ovaris bovins.

Gràcies al Dr. Carlos Barata, de qui he après molt del món de la ciència, al Dr. Demetrio Ráldua per les seves valuoses aportacions a aquest treball i al Dr. Joan Ribó per haver ampliat els meus coneixements especialment del assaig Microtox.

Gràcies a la Dra. Argelia Castaño per permetre'm realitzar la estada al Laboratori del CISA-INIA a Madrid i ensenyar-me les proves de citotoxicitat amb línies de peix. Moltes gràcies també a tot el seu equip, en especial a la Dra. Mª Teresa Llorente pel seu suport i disponibilitat durant la realització de la meva estada.

Molt especialment als meus companys de laboratori, al Dr. David López per ensenyar-me les tècniques bàsiques del cultiu de línies cel·lulars, a la Dra. Bettina Vallés per ensenyar-me els assaigs amb algues i peixos i en especial pels seus viatges en cotxe Terrassa-Barcelona, a la Joana Damaso per compartir amb mi els seus coneixements sobre biomarcadors, a la Esther Pérez per amenitzar les eternes hores compartides al transport públic, a l'Anna Cornellas per transmitir-me la seva alegria per la vida, a la Ana María, al Dr. Carles Gibert, a la Dra. Minerva García, a l'Esteban, Silvia Ribó, Laura, Anna Navarro i a tots els altres companys amb els quals he coincidit durant aquest anys al laboratori.

Gràcies també als amics pels ànims que sempre m'han donat i per les bones estones compartides i sobretot per escoltar-me. Esteu tots en aquest parell de línies. He d'agrair especialment a En Joseba per ajudar-me amb els seus fantàstics coneixements.

A la meva família i en especial als meus pares, pel seu recolzament, valentia i esforç perquè pogués realitzar els meus estudis universitaris lluny de casa, tot i sabent que seria molt temps fora de s' illa. Sense ells mai hagués arribat fins aquí. Gràcies també a les meves germanes, a Na Marta per la seva inestimable ajuda i a Na Nieves per donar-me ànims en les seves llargues converses telefòniques i gràcies a la meva àvia Francisca per confiar sempre en mi. Moltes gràcies també a Na Isabel, En Vicent i especialment a En Toni per la seva ajuda en les impresions.

I sobretot, al Israel, per la seva "infinita" paciència, la seva gran ajuda i especialment per estar al meu costat durant aquests darrers mesos.

Moltes gràcies a tothom!!

Lo que hacemos es como una gota de agua en el océano
pero sin esa gota de agua el océano estaría vacío.

Teresa de Calcuta

Abstract

0.1 Abstract

Carbofuran and chlorpyrifos are two well-known pesticides widely investigated, and its effects on different organisms have been previously reported in separate studies. For this reason were considered to be good model subtances, relevant from the environmental perspective. On the other hand, we selected this kind of compounds because they are used in many tones annually in agriculture and horticulture and they are significant especially in greenhouse-based production of vegetables and fruits in southern Europe, particularly Spain.

The general objective of this work was to compare the response of in vitro assays using cell lines with assays using fish in vivo in order to contribute to the development of alternative methods to the use of laboratory animals. Furthermore, we compared two types of cells, a fish cell line from an established culture and mammalian cells obtained from a primary cell culture, in order to see if there are similar responses based on common mechanisms of toxicity. A better knowledge of these mechanisms facilitates the interspecies extrapolation of the impact of environmental contaminants, which is one of the major challenges to ecotoxicologists.

In order to have a general point of view of the toxicity of these pesticides, single and in mixture, acute toxicity was evaluated using a battery of ecotoxicological model systems and indicators representative of a wide range of organisms. The systems studied included: RTG-2 fish cell line, primary cell cultures from bovine granulosa cells, the marine bacteria (Vibrio fischeri), three species of freshwater microalgae (Chlorella vulgaris, Scenedesmus subspicatus and Selenastrum capricornutum) and the vertebrates zebra fish (Danio rerio). Sublethal effects were also evaluated in both types of cells using several biomarkers such as assessment of the DNA damage as genotoxic indicator, inhibition of production of progesterone as indicator of the aromatase activity and inhibiton of acetylcholinesterase activity as indicator of exposure to pesticides measured also in zebra fish.

By comparing the obtained in vitro fish cell line and mammalian primary cell cultures results with currently used bacteria, algae and fish acute toxicity tests, it was possible to compare its sensitivity against these conventional toxicity tests.

As conclusions of the study we can say: 1) fish cell lines can be used as an alternative to the use of fish in laboratory; 2) the simultaneous use of in vitro fish cell lines with fish

species allow to assess whether cellular responses in vitro could mimic toxicity responses of individual fish, thus to directly assess the ecological relevance of the proposed in vitro cell based test; 3) cell lines are the most sensitive bioassay of the studied battery; 4) the use of a battery of multispecies tests to determine the toxicity of any product is recommended; 5) The aquatic test using bacteria or microalgae are quite interesting, but they cannot be considered as a substitute for the studies on fish, because of they assess the effect of toxicants on other trophic levels, not on fish and 6) synergistic and antagonistic toxicity effects were observed with pesticide cocktails relative to pure compound toxicities.

0.2 Resum

Carbofuran i chlorpyrifos són dos pesticides àmpliament investigats. Els seus efectes en diferents organismes han estat prèviament estudiats en diferents treballs. Per aquesta raó es va considerar que serien bones substàncies model, rellevants des de una perspectiva medi ambiental. Per altra banda, aquesta classe de compostos són utilitzats anualment en moltes tones en agricultura i horticultura i són especialment importants en la producció de vegetals i fruites en hivernacles en el sud d'Europa, particularment en Espanya.

L'objectiu general d'aquest treball és comparar la resposta de assaigs in vitro utilitzant cèl·lules amb assaigs in vivo utilitzant peixos, per tal de contribuir al desenvolupament de mètodes alternatius a l'ús d'animals de laboratori. A més a més dintre dels assaigs in vitro, és van comparar dos tipus de cèl·lules, una línia cel·lular establerta de peix i un cultiu primari de cèl·lules de mamífer, per veure les diferents respostes basades en mecanismes comuns de toxicitat. El fet d'assolir un millor coneixement d'aquestos mecanismes facilita l'extrapolació entre espècies per l'avaluació de l'impacte de contaminants medi ambientals, la qual cosa és un dels majors reptes dels ecotoxicolegs.

La toxicitat aguda d'aquestos pesticides, individuals i en barreja, es va avaluar emprant una barreja de sistemes i indicadors de models ecotoxicològics . Els sistemes estudiats inclouen: la línia cel·lular RTG-2 , cultius primaris de cèl·lules de la granulosa d'ovaris de bovins, la bactèria marina Vibrio fischeri, tres espècies de microalgues, Chlorella vulgaris, Scenedesmus susbspicatus i Selenastrum capricornutum i com a model de vertebrat, el peix zebrafish, Danio rerio. Els efectes subletals també van ser avaluats en els dos tipus cel·lulars utilitzant biomarcadors, tals com l'avaluació del dany a l'ADN com indicador genotòxic, la inhibició de la producció de progesterona com indicador de l'activitat de l'aromatasa i la inhibició de la activitat de l'enzim acetilcolinestarasa com indicador de l'exposició als pesticides la qual també es va mesurar en zebra fish.

Al comparar els resultats obtinguts amb la línia cel·lular de peix i els cultius primaris de mamífer amb els resultats dels tests de toxicitat aguda amb bactèries, microalgues i peixos, va estar possible comparar la sensibilitat de les cèl·lules respecte els test de toxicitat convencionals.

Es poden extreure les següents conclusions d'aquest estudi:1) les línies cel·lulars poden ser utilitzades com alternativa al ús de peixos en laboratori; 2) l'utilització simultània de línies cel·lulars derivades de peixos amb peixos in vivo permet avaluar si les respostes in vitro poden imitar les respostes de toxicitat obtingudes amb els peixos, així es permet avaluar la rellevància ecològica del test basat en cèl·lules in vitro; 3) les línies cel·lulars són el bioassaig més sensible dels utilitzats en aquesta bateria; 4) es recomana l'ús d'una bateria de test multiespècies per determinar la toxicitat de qualsevol producte; 5) els tests aquàtics que empren bactèries o microalgues són interessants però no poden considerar-se substitutius dels estudis amb peixos, perquè avaluen l'efecte dels tòxics a un altra nivell tròfic i ; 6) s'observen efectes toxicològics sinèrgics i antagonistes quan s'utilitzen les barreges de pesticides en front dels compostos purs.

0.3 Resumen

Carbofuran y chlorpyrifos son dos pesticidas ampliamente investigados. Sus efectos en diferentes organismos han sido previamente estudiados en diferentes trabajos. Por esta razón se considero que serian unas buenas sustancias modelo, relevantes desde una perspectiva medioambiental. Por otra parte, esta clase de compuestos son utilizados anualmente en muchas toneladas en agricultura y horticultura y son especialmente importantes en la producción de vegetales y frutas en invernaderos en e l sur de Europa, particularmente en España.

El objetivo general de este trabajo es comparar la respuesta de los ensayos in vitro utilizando células y los ensayos in vivo utilizando peces, para contribuir al desarrollo de métodos alternativos al uso de animales de laboratorio. Además dentro de los estudios in vitro se compararon dos tipos de células, una línea celular establecida de pez y un cultivo primario de células de mamífero, para ver las diferentes respuestas basadas en mecanismos comunes de toxicidad. El hecho de alcanzar un mejor conocimiento de estos mecanismos facilita la extrapolación entre especies para la evaluación del impacto de contaminantes medio ambientales, lo cual es uno de los mayores retos de los ecotoxicólogos.

La toxicidad aguda de estos pesticidas, individuales y en mezcla, se evaluó utilizando una mezcla de sistemas y indicadores de modelos ecotoxicológicos. Los sistemas estudiados incluyen: la línea celular RTG-2, cultivos primarios de células de granulosa de ovarios bovinos, la bacteria marina Vibrio fischeri, tres especies de microalgas, Chlorella vulgaris, Scenedesmus subspicatus y Selenastrum capricornutum como modelo de vertebrado, el pez zebra fish, Danio rerio. Los efectos subletales también fueron evaluados en los dos tipos celulares utilizando biomarcadores tales como la evaluación del daño al ADN como indicador geonotóxico, la inhibición de la producción de progesterona como indicador de la actividad aromatasa y la inhibición de la actividad del enzima acetilcolinesterasa como indicador de la exposición a los pesticidas la cual también se midió en zebra fish.

Al comparar los resultados obtenidos con la línea celular de pez y los cultivos primarios de mamífero con los resultados de los tests de toxicidad aguda con bacterias, microlagas y peces, fue posible comparar la sensibilidad de las células con los test de toxicidad convencionales.

Se pueden extraer las siguientes conclusiones de este estudio: 1) las líneas celulares pueden ser utilizadas como alternativa al uso de peces en laboratorio; 2) la utilización simultánea de líneas celulares derivadas de peces con los peces in vivo permite evaluar si las respuestas in vitro pueden imitar las respuestas de toxicidad obtenidas con los peces in vivo, así se permite evaluar la relevancia ecológica del test basado en células in vitro; 3) las líneas celulares son el bioensayo más sensible de los utilizados en esta batería; 4) se recomienda el uso de una batería de test multiespecies para determinar la toxicidad de cualquier producto; 5) los test acuáticos que utilizan bacterias o microalgas son interesantes pero no pueden considerarse substitutos de los estudios con peces, porque evalúan el efecto de los tóxicos a otro nivel trófico y; 6) se observan efectos toxicológicos sinérgicos y antagonistas cuando se utilizan las mezclas de pesticidas respecto los compuestos puros.

Introduction

1. Introduction

1.1. Concept of ecotoxicology

Ecotoxicology is a science that deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at different levels of biological organisation, from the molecular, cellular, tissue, organ and organism level, up to different populations and ecosystems. Knowledge of common mechanisms of toxicity facilitates the interspecies extrapolation of the impact of environmental contaminants, which is one of the major challenges to ecotoxicologists.

Ecotoxicology can be also described as a vast and complex discipline which employs various approaches for investigations, including residue analysis, biomarkers, field surveys, and toxicity testing.

Experimental model systems and bioassays are currently used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation and to register new chemicals as well as to investigate their effects and mechanisms of action. In addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment.

Most of the procedures used in regulatory and non-regulatory toxicology are carried out on mammals, but public pressure to minimize the use of vertebrates in ecotoxicity testing (Walker et al., 1998) and scientific interest in promoting the study of the effects of chemicals on both terrestrial organisms (including vegetables) and aquatic environments is growing. The use of invertebrate organisms, microorganisms, and plants—despite being far more abundant in nature than vertebrates—is still not well represented in ecotoxicology (Reppeto et al., 2001).

The response of a single toxicity assay is an insufficient measure of the adverse biological impact of a compound in a generally diverse receiving ecosystem. Different toxicants act differently and not all life forms are equally susceptible. Consequently, single bioassays will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be used (Bierkens et al., 1998, Isooma et al., 1995). A minimum ecotoxicological in vitro test battery should at least include bacteria, protozoans, algae, invertebrates fish cell lines, or cells isolated from fish and other species.

1.2. Pesticides

Pesticides enter waterways from agricultural and urban run-off, movement through soil into watercourses and after direct application (Schulz and Leiss, 1999) and may be transported to estuaries and coastal waters (Readman et al., 1992).

Consequently, wastewater from greenhouses and runoff from agricultural land are nearly always contaminated with pesticides. Because of their toxicity, pesticides affect the ecology of the receiving bodies of water and contaminate drinking water supplies. Pesticides pose various threats to organisms, including humans, and are thus a cause of concern.

Some pesticides bioaccumulate affecting fish, birds and other animals and appearing in human food sources. Depending on the local cultivation practices, a waterbody may receive a single pesticide or a varying cocktail of compounds. Toxicity of pesticides contaminated effluent depends on the amounts and types of the individual pesticides present; however, even for pure compounds, concentration–toxicity relationships are generally nonlinear. Cocktails of compounds pose bigger problems because toxicity of a mixture is not easily linked to individual toxicities of components in the mixture. Thus, for predicting the impact of a wastewater stream on the ecology of a receiving body such as a biological wastewater treatment facility, or a lake, the toxicity of the contaminated water needs to be determined with single and multiple contaminants. Few such data exist.

Human and animal exposure to chemicals is rarely limited to a single chemical. Individuals are exposed daily to a variety of chemicals in food, drink, cosmetics and indoor and outdoor pollutants. In recent years, various environmental problems have led to increased concern about potential toxicity from exposure to multiple chemicals, including pesticide residues detected in food or water (Yang et al., 1989). The use of pesticide combinations ensured that any synergistic toxicity effects were identified.

Moreover, the rapid increase in production and use of organophosphorous and carbamate pesticides has raised concerns about their potential to cause harm to human and non-target wildlife populations. The sublethal effects of pesticides on non-target aquatic organisms that may be essential to the health and maintenance of the vulnerable, highly productive ecosystems are the subjects of intense study by scientists

and environmental managers (Mineau, 1991; Roast et al., 1999; Werner et al., 2000; Doran et al., 2001; Fulton and Key, 2001).

1.2.1. Classification of pesticides

A pesticide or pesticide product is a chemical, organism or device used to control, prevent, destroy, repel, attract or reduce pests. Though often misunderstood to refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests.

By their nature, most pesticides create some risk of harm. Pesticides can cause harm to humans, animals, or the environment because they are designed to kill or otherwise adversely affect living organisms but at the same time, pesticides are useful to society. Pesticides can kill potential disease-causing organisms and control insects, weeds, and other pests.

Since 1992, several countries have been working on the development of a Globally Harmonized System (GHS) for the classification and labelling of chemicals. The GHS is a hazard-based system, which will encompass all hazardous chemicals, including pesticides. The GHS includes (1) harmonized criteria for evaluating the health, environmental and physical hazards of chemicals, and (2) harmonized hazard communication elements, including the requirements for labelling and for safety data sheets.

The pesticides can be classificated in different ways according to the Spanish legislation, in concret according to the RD 3349/83, of 30 of November about "Technical-sanitary reglamentation for the production, commercialization and application of pesticides" (BOE nº 20, 24 of January of 1984) modified by RD 162/1991, of 8 of February (BOE nº 40, of 15 of February of 1991).

According to the application use:

a. *Pesticides of phitosanitary use: applied on health and control of vegetals.*
b. *Pesticides of livestock use: applied on animals or related activities.*
c. *Pesticides of food industry use: applied on treatments of products or dispositives related with the food industry.*
d. *Pesticides of environmental use: applied on the sanitation of places or other public or privates sites.*
e. *Pesticides of personal hygiene use: applied directly on the people. (i.e., Insect repellents for personal use).*
f. *Pesticides of domestic use: whatever househod product prepared for being applicated by not qualified people. (i.e., Cockroach sprays and baits; Rat and other rodent poisons; Flea and tick sprays, powders and pet collars; Kitchen, laundry, and bath disinfectants and sanitizers; Products that kill mold and mildew; Some lawn and garden products, such as weed killers; Some swimming pool chemicals).*

According to their specific action, there are multiple classifications; one of the most used is the decimal one, which considers:

I. *Insecticides (i.e., products to control insects)*
II. *Miticides or acarides (i.e., products to control mites)*
III. *Fungicides (i.e., products to control fungus diseases)*
IV. *Nematocides, disinfectants (like bactericides, bacteriostats) and fumigants in general.*
V. *Herbicides (i.e., products to control weeds)*
VI. *Phitoregulators like algicides (i.e., products to control algae)*
VII. *Molluscicides (i.e., products to control slugs and snails), rodenticides (i.e., products to control rats and mice) and others.*
VIII. *Specifics for post-crops and seed treatment.*
IX. *Protectors of wood, fibers and other derivates*
X. *Specific pesticides in general (Avicides (i.e., products to control birds), Piscicides (i.e., products to control fish)*

The formulations could be classificated depending on the presentation state or the system used for their application; these characteristics determine the facility to penetrate in the exposed organism. According to these criteria, the next groups could be considered:

1. *Gases or liquidized gases*
2. *Fumigants and aerosols*
3. *Powder with a diameter inferior to 50 µm*
4. *Solids, except baits and presentations in tablet form*
5. *Liquids*
6. *Bait and tablets*

From the point of view of their chemical type, pesticides could be classificated in several groups; the most important are the following:

AS Arsenic compound
BP Bipyridylium derivative
C Carbamate
CO Coumarin derivative
CU Copper compound
HG Mercury compound
NP Nitrophenol derivative
OC Organochlorine compound
OP Organophosphorus compound
OT Organotin compound
PAA Phenoxyacetic acid derivative
PZ Pyrazole
PY Pyrethroid
T Triazine derivative
TC Thiocarbamate

These chemical classifications are included only for convenience, and do not represent a recommendation of the part of the World Health Organization (WHO) as to the way in which the pesticides should be classified. It should, furthermore, be understood that some pesticides may fall into more than one type.

Nowadays, the Biologically-based pesticides, such as pheromones and microbial pesticides like Bacillus thuringiensis, are becoming increasingly popular and often are safer than traditional chemical pesticides. In addition, the Environmental Protection Agency (EPA) is registering reduced-risk conventional pesticides in increasing numbers.

Attending to their risk degree for the people, pesticides are classificated according to the RD 3349/83 in the next way:

1. According to the **toxicity degree**, *there are the next cattegories:*

a. **Low Risk***: don't produce appreciable risks caused by inhalation, swallowing and/or cutaneous penetration*

b. **Harmful***: may produce risks of limited seriousness caused by inhalation, swallowing and/or cutaneous penetration*

c. **Toxic***: may produce seriuous risks, acute or chronic, included death, caused by inhalation, swallowing and/or cutaneous penetration*

d. **Very toxic***: may produce extremely serious risks, acute or chronic, included death, caused by inhalation, swallowing and/or cutaneous penetration*

2. According to **another effects***:*

a. **Corrosives***: in contact with live tissues could produce them a destructive action*

b. **Irritant***: the no corrosives that by direct contact extended or repeated with the skin or the mucous could cause an inflamatory reaction*

c. **Easily inflammable***: The pesticides that:*

- *At normal temperature and without an aport of energy in open spaces could be heated and even be inflammated.*
- *In solid state, could be inflammated easily for the brief action of an inflammable source and continue burning after remove the inflammable source.*
- *In liquid state, with an inflammation point lower than 21ºC.*
- *Gaseous those are inflammables in open spaces at normal pressure.*
- *The ones that in contact with water or wet air give off gases easily inflammables in dangerous quantities.*

d. **Explosives***: the ones that could explode under the effect of a flame o which are more sensitive to crashes or friction than dinitrobencene.*

The toxicological classification of the pesticides in the categories of low risk, harmful, toxic or very toxic is carried out attending basically to their acute toxicity expressed as LD50 (the median lethal dose of the formulated pesticide product. The lower the LD50 value of the product, the more acutely toxic the product is) by oral or dermal administration for rats or as CL50 (the median lethal concentration of the formulated pesticide product. The lower the LC50 value of a chemical the more acutely toxic that chemical is by inhalation for rats, according to several criterias specified in the RD 3349/83.

The pesticides need a human health risk assessment based on a thorough understanding of the toxicological profile of the pesticide and the nature and relevance of the observed toxic effects to human health. Standardized studies in test animals are required. These tests provide the basis for determining what is the toxic effect; at what dose level does it appear; and at what dose level is there no effect. Short and long-term toxicity studies as well as studies in special areas, such as carcinogenicity, mutagenicity, etc., are assessed. Occupational exposure assessments determine the exposure that could occur for persons handling the pesticide on a regular basis, any possible bystander exposure and the potential for exposure from food residues. There is necessary to take special consideration of children and other vulnerable groups, and the need to assess aggregate exposure and cumulative effects of pesticides that act in the same way.

It is convenient to perform an environmental control by means of the quantification of the compound in the air and the comparation of the founded concentrations with maximum values of reference destined to protect the workers health.

Between these values, the most accepted are the proposed by the ACGIH (American Conference of Governmental Industrial Hygienists) as Threshold limit value (TLV)®, means the maximum airborne concentration of a substance to which the (ACGIH) believes that nearly all workers may be repeatedly exposed day after day without adverse effects.

The TLV® is divided into three categories: TLV-Time-Weighted Average® (TLVTWA ®), TLV-Short-Term Exposure Limit® (TLV-STEL®), and TLV-Ceiling® (TLV-C®).
"TLV-TWA®" means the time-weighted average concentration for a normal eight-hour workday and a 40-hour workweek, to which nearly all workers may be repeatedly exposed, day after day, without adverse effect (as defined in the ACGIH Handbook).

"TLV-STEL®" means the concentration to which workers may be exposed continuously for a short period of time without suffering from irritation, chronic or irreversible tissue damage, or narcosis of sufficient degree to increase the likelihood of accidental injury, impair self-rescue or materially reduce work efficiency.

The TLV-STEL® supplements the TLV-TWA® where there are recognized acute effects from a substance whose toxic effects are primarily of a chronic nature.

The environmental control make possible to consider the exposure risk depending on the toxic concentration in air supposing that the penetration in the organism occurs only by inhalation, but in the case of the pesticides, the possibility of entrance by other ways is also possible. For this reason could be more interesting to obtain information of the true level of personal exposure through a biological control.

This control could consist of the direct determination of the pesticide or its metabolites in a biological fluid; that is the case of a lot of organochlorated pesticides like DDT, Dieldrin or Lindane in blood or the pentaclorphenol in urine, as well as the p-nitrophenol, 1-naphtol or 2-isopropoxiphenol in urine, metabolites of Parathion, Carbaril or Baygón, respectively.

The founded concentrations are compared with biological exposure index values (BEI). These kinds of tests are selective respect to the pollutant, but for some families of pesticides, in concret organophosphates and carbamates are possible to do a non selective biological control. It consists of the measure of the level of inhibition of the acetylcholinesterase activity.

1.2.2. CARBOFURAN

Carbofuran (Figure 1) is a broad-spectrum carbamate pesticide that kills insects, mites, and nematodes on contact or after ingestion. It is used against soil and foliar pests of field, fruit, vegetable, and forest crops.

Carbamates are insecticides used in crop protection for their low persistence, wide action spectra and ability to control pests. As with other chemicals, ecotoxicity studies are specially needed in order to know its effects on the aquatic and terrestrial organisms and their potential risk as environmental pollutant (Butler, 1977; Antón et al, 1990).

Carbofuran is available in liquid and granular formulations, but the granule form is banned in the U.S. The EPA initiated a ban on all granular formulations of carbofuran, which became effective on September 1, 1994. Before 1991, 80% of the total usage of carbofuran was in granular formulations. The ban was established to protect birds and is not related to human health concerns. Bird kills have occurred when birds ingested carbofuran granules, which resemble grain seeds and when predatory or scavenging birds have ingested small birds or mammals, which had eaten carbofuran pellets. There is no ban on liquid formulations of carbofuran. Liquid formulations bear the Signal Word WARNING. Granular formulations bear the Signal Word DANGER. According to the UNE directives formulations of carbofuran are classified as very toxic. Trade and Other Names: Trade names include Furadan, Bay 70143, Carbodan, Carbosip, Chinofur, Curaterr, D 1221, ENT 27164, Furacarb, Kenafuran, Pillarfuron, Rampart, Nex, and Yaltox. Chemical Class: carbamate.

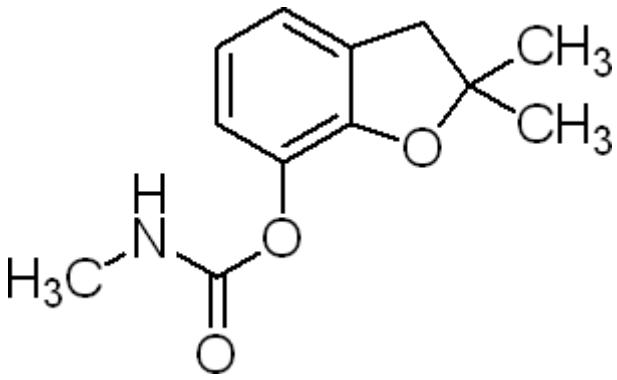


Figure 1. Chemical structure of carbofuran

Physical Properties*:*

Appearance: *Carbofuran is an odorless, white crystalline solid. Heat breakdown can release toxic fumes. Fires, and the runoff from fire control, may produce irritating or poisonous gases. Closed spaces (storage, etc.) should be aired before entering.*

Chemical Name: *2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate*

CAS Number: *1563-66-2*

Molecular Weight: *221.25*

Water Solubility: *320 mg/L at 25 ºC (Kidd et al., 1991)*

Solubility in Other Solvents: *acetone; acetonitrile; benzene; cyclohexone (Baron et al., 1991).*

Melting Point: *153-154 ºC (Kidd et al., 1991)*

Vapor Pressure: *2.7 mPa at 33 ºC (Kidd et al., 1991)*

Partition Coefficient: *1.2304-1.4150 (Kidd et al., 1991)*

Adsorption Coefficient: *22 (Wauchope et al., 1992)*

Toxicological Effects:

Acute toxicity: *Carbofuran is highly toxic by inhalation and ingestion and moderately toxic by dermal absorption. As with other carbamate compounds, carbofuran's cholinesterase-inhibiting effect is short-term and reversible. Symptoms of carbofuran poisoning include: nausea, vomiting, abdominal cramps, sweating, diarrhea, excessive salivation, weakness, imbalance, blurring of vision, breathing difficulty, increased blood pressure, and incontinence. Death may result at high doses from respiratory system failure associated with carbofuran exposure. Complete recovery from an acute poisoning by carbofuran, with no long-term health effects, is possible if exposure ceases and the victim has time to regain their normal level of cholinesterase and to recover from symptoms. The oral LD50 is 5 to 13 mg/kg in rats, 2 mg/kg in mice, and 19 mg/kg in dogs. The dermal LD50 is >1000 mg/kg in rabbits. The LC50 (4-hour) for inhalation of carbofuran is 0.043 to 0.053 mg/L in guinea pigs (Baron et al., 1991; Kidd et al., 1991)*

Chronic toxicity: *Rats given very high doses (5 mg/kg/day) for two years showed decreases in weight. Similar tests with mice gave the same results. Prolonged or*

repeated exposure to carbofuran may cause the same effects as an acute exposure (Baron et al., 1991).

Reproductive effects: *Consuming high doses over long periods of time caused damage to testes in dogs, but carbofuran did not have any reproductive effects on rats or mice (Baron et al., 1991). Available studies indicate carbofuran is unlikely to cause reproductive effects in humans at expected exposure levels.*

Teratogenic effects: *Studies indicate carbofuran is not teratogenic. No significant teratogenic effects have been found in offspring of rats given carbofuran (3 mg/kg/day) on days 5 to 19 of gestation. No effects were found in offspring of mice given as much as 1 mg/kg/day throughout gestation. In rabbits, up to 1 mg/kg/day on days 6 to 18 of gestation was not teratogenic (Baron et al., 1991).*

Mutagenic effects: *Weak or no mutagenic effects have been reported in animals and bacteria. Carbofuran is most likely nonmutagenic (Baron et al., 1991).*

Carcinogenic effects: Data from animal studies indicate that carbofuran does not pose a risk of cancer to humans (Baron et al., 1991).

Organ toxicity: *Carbofuran causes cholinesterase inhibition in both humans and animals, affecting nervous system function.*

Fate in humans and animals: Carbofuran is poorly absorbed through the skin (Gosselin et al., 1984). It is metabolized in the liver and eventually excreted in the urine. The half-life in the body is from 6 to 12 hours. Less than 1% of a dose will be excreted in a mother's milk. It does not accumulate in tissue (Baron et al., 1991).

Ecological Effects:

The environmental assessment includes evaluation of environmental chemistry, fate, persistence and toxicity to fish and wildlife. To assess the potential effects of pesticides on the environment, tests in surrogate species together with conservative estimates of expected environmental concentrations, are used. The environmental assessment predicts potential risks for a wide range of wildlife species, and ecosystems under a wide range of conditions. The environmental assessment also results in recommendations on mitigative measures that would reduce concerns for residue accumulation, groundwater contamination, runoff and risk to non-target organisms.

Effects on birds: *Carbofuran is highly toxic to birds. One granule is sufficient to kill a small bird. Bird kills have occurred when birds ingested carbofuran granules, which resemble grain seeds in size and shape, or when predatory or scavenging birds have ingested small birds or mammals that have eaten carbofuran pellets (EPA, 1991).*

Red-shouldered hawks have been poisoned after eating prey from carbofuran-treated fields (Smith et al., 1992).

The LD50 is 0.238 mg/kg in fulvous ducks, 0.48 to 0.51 mg/kg in mallard ducks, 12 mg/kg in bobwhite quail, and 4.15 mg/kg in pheasant (Smith et al., 1992). The LD50 is 25 to 39 mg/kg in chickens consuming carbofuran as a powder (Kidd et al., 1991). The LC50 (96-hour) in Japanese quail is 746 ppm (Hill et al., 1986).

Effects on aquatic organisms: *Carbofuran is highly toxic to many fish. The LD50 (96-hour) is 0.38 mg/L in rainbow trout and 0.24 mg/L in bluegill sunfish (Kidd et al., 1991). The compound has a low potential to accumulate in aquatic organisms. The bioconcentration factor ranges from 10 in snails to over 100 in fish (Howard et al., 1991).*

Effects on other organisms: *Carbofuran is toxic to bees except in the granular formulation (Kidd et al., 1991).*

Environmental Fate:

Breakdown in soil and groundwater: *Carbofuran is soluble in water and is moderately persistent in soil. Its half-life is 30 to 120 days. In soil, carbofuran is degraded by chemical hydrolysis and microbial processes. Hydrolysis occurs more rapidly in alkaline soils (Howard et al., 1991). Carbofuran breaks down in sunlight. Carbofuran has a high potential for groundwater contamination (Howard et al., 1991). Carbofuran is mobile to very mobile in sandy loam, silty clay, and silty loam soils; moderately mobile in silty clay loam soils; and only slightly mobile in muck soils. Small amounts of carbofuran have been detected (1 to 5 ppb) in water table aquifers beneath sandy soils in New York and Wisconsin (Howard et al., 1991).*

Breakdown in water: *In water, carbofuran is subject to degradation by chemical hydrolysis under alkaline conditions. Photodegradation and aquatic microbes may also contribute to degradation. The hydrolysis half-lives of carbofuran in water at 25ºC are 690 – 8.2 – 1.0 weeks at pH values of 6.0 - 7.0 - and 8.0, respectively. Carbofuran does not volatilize from water, nor adsorb to sediment or suspended particles (Howard et al., 1991).*

Breakdown in vegetation: *The half-life of carbofuran on crops is about 4 days when applied to roots, and longer than 4 days if applied to the leaves***.**

1.2.3. CHLORPYRIFOS

The organic pesticide, chlorpyrifos (Figure 2), introduced in 1965, is one of the most widely used chemical organophosphate insecticides in the market today (Hayes and Laws, 1990).

Chlorpyrifos is a broad-spectrum organophosphate insecticide. While originally used primarily to kill mosquitoes, it is no longer registered for this use. Chlorpyrifos is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton, fruit, nut and vegetable crops, and well as on lawns and ornamental plants. It is also registered for direct use on sheep and turkeys, for horse site treatment, dog kennels, domestic dwellings, farm buildings, storage bins, and commercial establishments. Chlorpyrifos acts on pests primarily as a contact poison, with some action as a stomach poison. It is available as granules, wettable powder, dustable powder and emulsifiable concentrate. About 40 million kg of chlorpyrifos is manufactured per year and it is an active ingredient in about 800 products in the US. During the mid-1990s, 4–5.5 million kg were used annually in non-agricultural situations in over 17% of households. Agricultural usage estimates vary even more with annual application somewhere between 4.5–10 million kg. Recently, the Environmental Protection Agency (EPA) and the manufacturers of chlorpyrifos have agreed to eliminate nearly all-household applications of the insecticide, but agriculture use continues.

According to the UNE directives Chlorpyrifos is classified as toxic. Products containing chlorpyrifos bear the Signal Word WARNING or CAUTION, depending on the toxicity of the formulation.

Trade and Other Names: *Trade names include Brodan, Detmol UA, Dowco 179, Dursban, Empire, Eradex, Lorsban, Paqeant, Piridane, Scout, and Stipend.*
Chemical Class: organophosphate

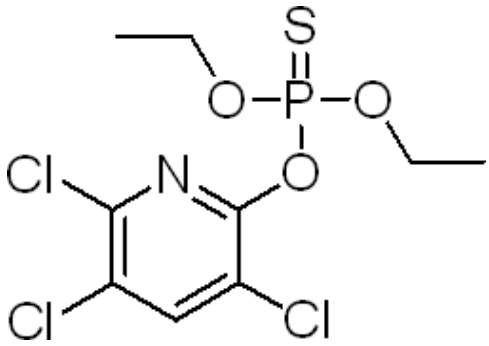


Figure 2. Chemical structure of chlorpyrifos

Physical Properties:

Appearance: *Technical chlorpyrifos is amber to white crystalline solid with a mild sulfur odor (Kidd et al., 1991)*
Chemical Name: *O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phosphorothioate (Kidd et al., 1991)*
CAS Number: *2921-88-2*
Molecular Weight: *350.62*
Water Solubility: *2 mg/L at 25 ºC (Kidd et al., 1991)*
Solubility in Other Solvents: *benzene; acetone; chloroform; carbon disulfide; diethyl ether; xylene; methylene chloride; methanol (Kidd et al., 1991***)**
Melting Point: *41.5-44 ºC (Kidd et al., 1991)*
Vapor Pressure: *2.5 mPa at 25 ºC (Kidd et al., 1991)*
Partition Coefficient: *4.6990 (Kidd et al., 1991)*
Adsorption Coefficient: *6070 (Wauchope et al., 1992)*

Toxicological Effects:

Acute toxicity: *Chlorpyrifos is moderately toxic to humans (EPA, 1989). Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant (Gallo et al., 1991). While some organophosphates are readily absorbed through the skin, studies in humans suggest that skin absorption of chlorpyrifos is limited (Gallo et al., 1991). Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, incoordination, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and slow heartbeat. Very high doses may result in unconsciousness, incontinence, and convulsions or fatality. People with respiratory ailments, recent exposure to cholinesterase inhibitors, cholinesterase impairment, or liver malfunction are at increased risk from exposure to chlorpyrifos. Some organophosphates may cause delayed symptoms beginning 1 to 4 weeks after an acute exposure, which may or may not have produced immediate symptoms (Gallo et al., 1991). In such cases, numbness, tingling, weakness, and cramping may appear in the lower limbs and progress to incoordination and paralysis. Improvement may occur over months or years, and in some cases residual impairment will remain (Gallo et al., 1991). Plasma cholinesterase levels activities have been shown to be inhibited when chlorpyrifos particles are inhaled. The oral LD50 for chlorpyrifos in rats is 95 to 270 mg/kg (Gallo et al., 1991; Kidd et al., 1991). The LD50 for chlorpyrifos is 60 mg/kg in mice, 1000 mg/kg in rabbits, 32 mg/kg in chickens, 500 to 504 mg/kg in guinea pigs, and 800 mg/kg in sheep (Gallo et al., 1991; Kidd et al., 1991; Gosselin et al., 1984). The dermal LD50 is greater than 2000 mg/kg in rats, and 1000 to 2000 mg/kg in rabbits (Gallo et al., 1991; Kidd et al., 1991; Dow Chemical Co, 1986). The 4-hour inhalation LC50 for chlorpyrifos in rats is greater than 0.2 mg/L (Dow Chemical Co, 1992).*

Chronic toxicity: *Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking, and drowsiness or insomnia. An influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported. When technical*

chlorpyrifos was fed to dogs for 2 years, increased liver weight occurred at 3.0 mg/kg/day. Signs of cholinesterase inhibition occurred at 1 mg/kg/day. Rats and mice given technical chlorpyrifos in the diet for 104 weeks showed no adverse effects other than cholinesterase inhibition (EPA, 1989). Two-year feeding studies using doses of 1 and 3 mg/kg/day of chlorpyrifos in rats showed moderate depression of cholinesterase. Cholinesterase levels recovered when the experimental feeding was discontinued (Gallo et al., 1991). Identical results occurred in a 2-year feeding study with dogs. No long-term health effects were seen in either the dog or rat study (Gallo et al., 1991). A measurable change in plasma and red blood cell cholinesterase levels was seen in workers exposed to chlorpyrifos spray. Human volunteers who ingested 0.1 mg/kg/day of chlorpyrifos for 4 weeks showed significant plasma cholinesterase inhibition.

Reproductive effects: *Current evidence indicates that chlorpyrifos does not adversely affect reproduction. In two studies, no effects were seen in animals tested at dose levels up to 1.2 mg/kg/day. No effects on reproduction occurred in a three-generation study with rats fed dietary doses as high as 1 mg/kg/day (EPA, 1989). In another study in which rats were fed 1.0 mg/kg/day for two generations, the only effect observed was a slight increase in the number of deaths of newborn offspring (Gallo et al., 1991).*

Teratogenic effects: *Available evidence suggests that chlorpyrifos is not teratogenic. No teratogenic effects in offspring were found when pregnant rats were fed doses as high as 15 mg/kg/day for 10 days. When pregnant mice were given doses of 25 mg/kg/day for 10 days, minor skeletal variations and a decrease in fetal length occurred (EPA, 1991; Dow Chemical Co, 1986). No birth defects were seen in the offspring of male and female rats fed 1.0 mg/kg/day during a three-generation reproduction and fertility study (Gallo et al. 1991).*

Mutagenic effects: *There is no evidence that chlorpyrifos is mutagenic. No evidence of mutagenicity was found in any of four tests performed (EPA, 1989).*

Carcinogenic effects: *There is no evidence that chlorpyrifos is carcinogenic. There was no increase in the incidence of tumors when rats were fed 10 mg/kg/day for 104 weeks, nor when mice were fed 2.25 mg/kg/day for 105 weeks (EPA, 1989).*

Organ toxicity: *Chlorpyrifos primarily affects the nervous system through inhibition of cholinesterase, an enzyme required for proper nerve functioning.*

Fate in humans and animals: *Chlorpyrifos is readily absorbed into the bloodstream through the gastrointestinal tract if it is ingested, through the lungs if it is inhaled, or through the skin if there is dermal exposure. In humans, chlorpyrifos and its principal metabolites are eliminated rapidly (Gallo et al., 1991). After a single oral dose, the half-life of chlorpyrifos in the blood appears to be about 1 day. Chlorpyrifos is eliminated primarily through the kidneys. Following oral intake of chlorpyrifos by rats, 90% is removed in the urine and 10% is excreted in the feces (Kidd et al., 1991). It is detoxified quickly in rats, dogs, and other animals. The major metabolite found in rat urine after a single oral dose is trichloropyridinol (TCP). TCP does not inhibit cholinesterase and it is not mutagenic. Chlorpyrifos does not have a significant bioaccumulation potential. Following intake, a portion is stored in fat tissues but it is eliminated in humans, with a half-life of about 62 hours (Gallo et al., 1991). When chlorpyrifos (Dursban) was fed to cows, unchanged pesticide was found in the feces, but not in the urine or milk (EPA, 1984). However, it was detected in the milk of cows for 4 days following spray dipping with a 0.15% emulsion. The maximum concentration in the milk was 0.304 ppm (Gallo et al., 1991). In a rat study, chlorpyrifos did not accumulate in any tissue except fat.*

Ecological Effects:

Effects on birds: *Chlorpyrifos is moderately to very highly toxic to birds (EPA. 1989). Its oral LD50 is 8.41 mg/kg in pheasants, 112 mg/kg in mallard ducks, 21.0 mg/kg in house sparrows, and 32 mg/kg in chickens (Kidd et al., 1991; EPA, 1989). The LD50 for a granular product (15G) in bobwhite quail is 108 mg/kg (Kidd et al., 1991; EPA, 1989). At 125 ppm, mallards laid significantly fewer eggs (EPA, 1989). There was no evidence of changes in weight gain, or in the number, weight, and quality of eggs produced by hens fed dietary levels of 50 ppm of chlorpyrifos.*

Effects on aquatic organisms: *Chlorpyrifos is very highly toxic to freshwater fish, aquatic invertebrates and estuarine and marine organisms (EPA, 1989). Cholinesterase inhibition was observed in acute toxicity tests of fish exposed to very low concentrations of this insecticide. Application of concentrations as low as 0.01 pounds of active ingredient per acre may cause fish and aquatic invertebrate deaths (EPA, 1989). Chlorpyrifos toxicity to fish may be related to water*

temperature. The 96-hour LC50 for chlorpyrifos is 0.009 mg/L in mature rainbow trout, 0.098 mg/L in lake trout, 0.806 mg/L in goldfish, 0.01 mg/L in bluegill, and 0.331 mg/L in fathead minnow (EPA, 1986). When fathead minnows were exposed to Dursban for a 200-day period during which they reproduced, the first generation of offspring had decreased survival and growth, as well as a significant number of deformities. This occurred at approximately 0.002 mg/L exposure for a 30-day period. Chlorpyrifos accumulates in the tissues of aquatic organisms. Studies involving continuous exposure of fish during the embryonic through fry stages have shown bioconcentration values of 58 to 5100 (Racke, 1992). Due to its high acute toxicity and its persistence in sediments, chlorpyrifos may represent a hazard to sea bottom dwellers (Schimmel et al., 1983). Smaller organisms appear to be more sensitive than larger ones (EPA, 1986).

Effects on other organisms*: Aquatic and general agricultural uses of chlorpyrifos pose a serious hazard to wildlife and honeybees (Kidd et al., 1991; EPA, 1984).*

Environmental Fate:

Breakdown in soil and groundwater: *Chlorpyrifos is moderately persistent in soils. The half-life of chlorpyrifos in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year, depending on the soil type, climate, and other conditions (Howard et al., 1991; Wauchope et al., 1992). The soil half-life of chlorpyrifos was from 11 to 141 days in seven soils ranging in texture from loamy sand to clay and with soil pHs from 5.4 to 7.4. Chlorpyrifos was less persistent in the soils with a higher pH (Racke et al., 1992). Soil half-life was not affected by soil texture or organic matter content. In anaerobic soils, the half-life was 15 days in loam and 58 days in clay soil (EPA, 1989). Adsorbed chlorpyrifos is subject to degradation by UV light, chemical hydrolysis and by soil microbes. When applied to moist soils, the volatility half-life of chlorpyrifos was 45 to 163 hours, with 62 to 89% of the applied chlorpyrifos remaining on the soil after 36 hours (Racke et al., 1992). In another study, 2.6 and 9.3% of the chlorpyrifos applied to sand or silt loam soil remained after 30 days (Racke et al., 1992). Chlorpyrifos adsorbs strongly to soil particles and it is not readily soluble in water (Wauchope et al., 1992; Racke et al., 1992). It is therefore immobile in soils and unlikely to leach or to contaminate groundwater (Racke et al., 1992). TCP, the principal metabolite of chlorpyrifos, adsorbs weakly to soil particles and appears to be moderately mobile and persistent in soils (EPA, 1989).*

Breakdown in water: *The concentration and persistence of chlorpyrifos in water will vary depending on the type of formulation. For example, a large increase in chlorpyrifos concentrations occurs when emulsifiable concentrations and wettable powders are released into water. As the pesticide adheres to sediments and suspended organic matter, concentrations rapidly decline. The increase in the concentration of insecticide is not as rapid for granules and controlled release formulations in the water, but the resulting concentration persists longer (EPA, 1986). Volatilization is probably the primary route of loss of chlorpyrifos from water. Volatility half-lives of 3.5 and 20 days have been estimated for pond water (Racke et al., 1992). The photolysis half-life of chlorpyrifos is 3 to 4 weeks during midsummer in the U.S. Its change into other natural forms is slow (Schimmel et al., 1983). Research suggests that this insecticide is unstable in water, and the rate at which it is hydrolyzed increases with temperature, decreasing by 2.5- to 3-fold with each 10 ºC drop in temperature. The rate of hydrolysis is constant in acidic to neutral waters, but increases in alkaline waters. In water at pH 7.0 and 25 ºC, it had a half-life of 35 to 78 days (Howard et al., 1991).*

Breakdown in vegetation: *Chlorpyrifos may be toxic to some plants, such as lettuce (McEwen et al. 1979). Residues remain on plant surfaces for approximately 10 to 14 days. Data indicate that this insecticide and its soil metabolites can accumulate in certain crops.*

1.3. In vitro methods

1.3.1. Alternative methods to animal experimentation

In 1959, Russell and Burch in their book, The Principles of Humane Experimental Technique, formally proposed the "3 R's" concept: reduction, refinement, and replacement.

Since then, alternative methods to experimentation with highly sensitive animals, particularly mammals, have been developed and some have been introduced in toxicology.

The recognition that a precise LD50 value for mammals is not required for notification of new chemicals leads to the development of alternative methods to the traditional LD50 test for acute oral toxicity determination. Some of these methods (fixed dose procedure, acute toxic class method, and up-and-down procedure) have now been accepted for regulatory purposes and must be used preferentially to the LD50 test.

1.3.2. The use of cells cultures

Over the last three decades, cell and tissue culture methods have been refined and have now become an essential tool in environmental research.

There are a lot of ethical, technical, scientific and economical reasons that support the development of in vitro methods for use in ecotoxicology. However, the regulatory authorities are reluctant to use cell-based in vitro tests in the context of ecotoxicology.
In vitro systems in ecotoxicology are expected not only to allow for extrapolation from in vitro effects to toxic effects in vivo, but also to provide information on biological responses at the supraindividual and ecological levels.

In ecotoxicological research, cellular effect studies are as important as studies in laboratory species because the primary interaction between chemicals and biota occurs at the surface of or in cells (Fent, 2001).

Cells, as the basic building blocks of all life forms, represent a key level of organisation for detecting and understanding common and unique mechanisms of toxicity.

Although in vitro assays may not always reflect the true in vivo situation, they undoubtedly provide important data on the activity of different compounds at the cellular level.

Interactions between chemical contaminants and biological systems initially take place at the cellular level. The use of in vitro cell cultures for ecotoxicological assessment can therefore be a valuable tool for the early and sensitive detection of chemical exposure (Segner and Braunbeck, 1998).

Cell cultures provide the best experimental system for studying toxic mechanisms at the molecular and cellular levels, by allowing cells to be studied in a controlled environment and in isolation from the multiple physiological systems, which regulate their activities in vivo.
In vitro systems share the characteristic that they exclude the influence of other organs and the circulatory and immune system, thus providing the possibility to study direct effects on a cell population (Figure 3).

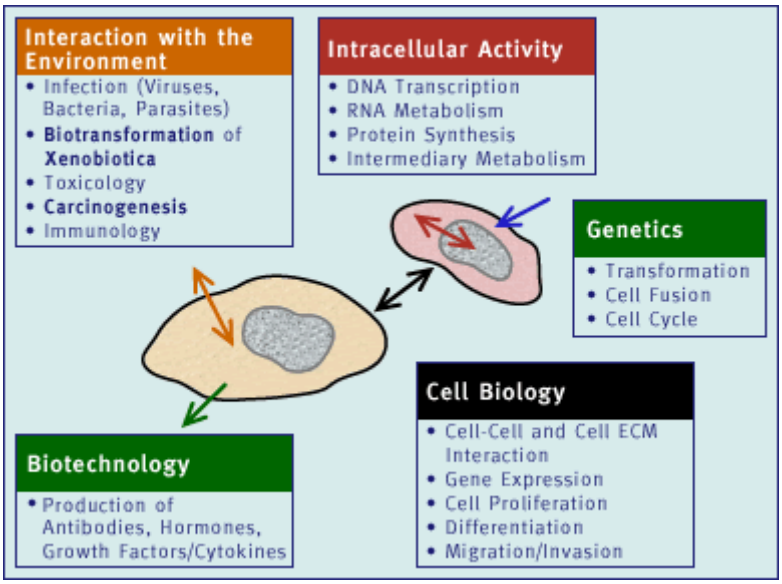


Figure 3. Cellular interactions with the environment, intracellular activity, biotechnology, genetics and cell biology.

Moreover, cells permit studies on a species that might not otherwise be studied, because that species cannot be maintained in a laboratory setting and/or are not routinely available.
Animal cell cultures permit the comparison of species at the cellular level under equivalent conditions of toxicant exposure. This is important for understanding the

relative potencies of toxicants in different species. Animal cell cultures can be used as a rapid, inexpensive screening tool to evaluate the toxicities of large numbers of individual compounds and of samples from the environment. This will probably become even more important in the future, as the new concepts of genomics and proteomics become incorporated into screening tests.

Genomics is based upon the application of DNA microarrays that allow the expression of hundreds to many thousands of genes to be monitored simultaneously, thus providing a broad and integrated picture of the way an organism responds to a changing environment (Gracey et al., 2001).

The entire protein complement of the genome, the 'proteome', can now be analyzed for changes associated with specific treatments, using 'peptide mass profiling', a combination of two-dimensional gel electrophoresis and mass spectrometry (Shepard et al., 2000). Proteomics research provided certain protein expression signatures (PESs), which are specific sets of proteins, present or absent, indicating specific toxicity profiles.

Cellular test systems will provide the basis for the application of automated and high-throughput technologies in ecotoxicological hazard assessment.

The application of animal cell cultures for the above purposes reduces the use of whole animals in toxicity testing, which is a goal supported by popular opinion in many countries around the world. As in human toxicology, the objective is the replacement, reduction or refinement of in vivo tests. The existing regulatory tests in ecotoxicology are in vivo tests for measuring organismic -- not ecological -- responses, such as death, growth, and reproduction. The special challenge for in vitro studies in ecotoxicology is the development of cellular test systems that not only can replace whole animal tests, but also have meaning at the ecological level. (Castaño et al., 2003).

Mammalian and fish-derived cell lines have been frequently employed in short-term cytotoxicity assays (Riva et al., 2005, 1994, López et al., 1998; Alañón et al., 2005, 2004, 2003, 2001, Stacchezzini et al., 2006). Extrapolation of in vitro toxicity results to the in vivo situation, based on absolute toxicity values may differ significantly depending on the toxicant, however, good correlations have been determined based on the relative ranking order of pollutants to fish cells with their water-borne in vivo toxicity

to live fish (Bols et al., 1985; Dierickx and van de Vyver, 1991; Segner and Lenz, 1993; Castaño et al., 1996). Consequently, in vitro toxicity tests clearly have an important role to play in providing appropriate data for the safety assessment of chemicals. Fish cells lines in particular, have proven to be valuable, rapid and cost-effective tools in the preliminary ecotoxicological evaluation of xenobiotics (Fent, 2001). Cells from various tissues and fish species have been used for toxicological assessment of chemicals and environmental samples (Bols et al., 1985; Kocan et al., 1985; Castaño et al., 1995a, 1995b; Kammann et al., 2001).

1.3.3. Primary cell cultures vs established cell lines

Two types of cell culture should be distinguished:

1. Primary cultures are obtained directly from animals and can keep the differentiated state for a short period (days to weeks). Functionally differentiated primary cell cultures have a limited life span, maintain differentiated properties and it has been improved by additives to the culture medium, components of the extracellular matrix or by different forms of co-culture (see Figures 4a-b), cell specific functions will eventually decline.

2.Established cell lines derive from primary cultures of cells from organs and tissues of different animal species by transformation processes, which are either spontaneous or induced by viruses, chemical or physical agents, or derived from tumoral tissues. Permanent cultures (e.g. HeLa, 3T3, RTG-2) have an unlimited proliferation capacity

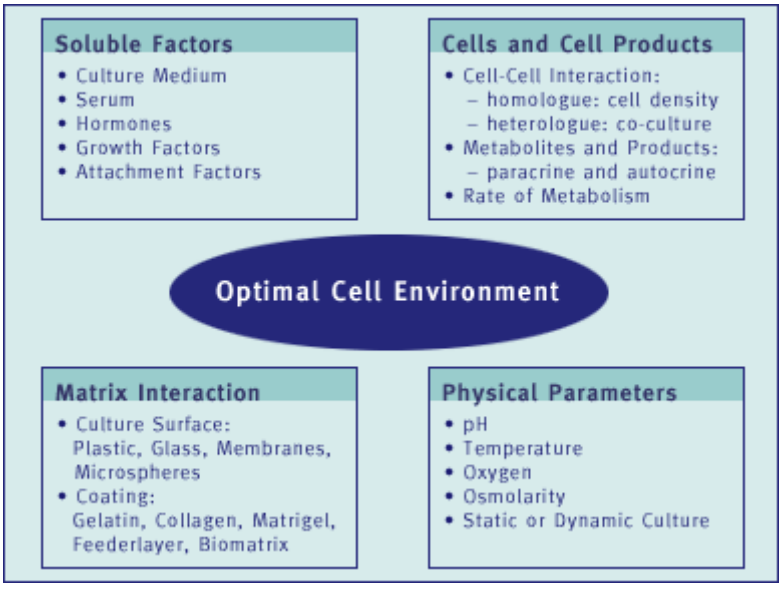


Figure 4a. Conditions for an optimal cell environment

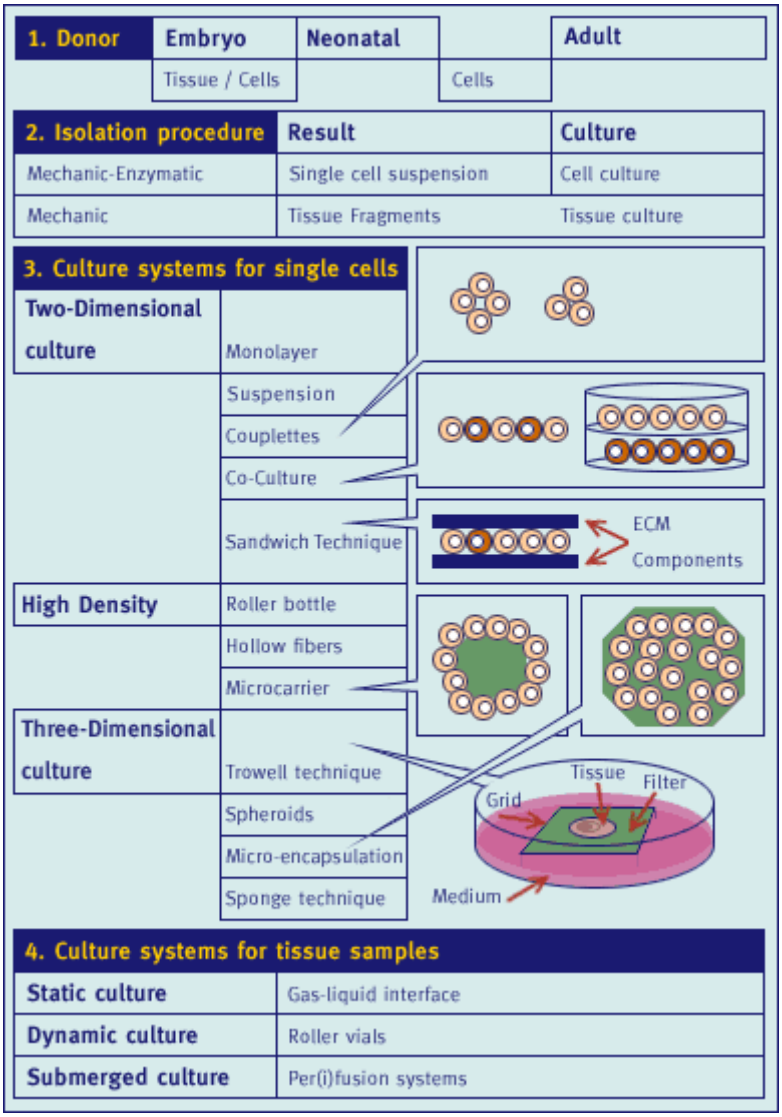


Figure 4b. Methods for obtention of cellular systems.

Primary cells cultures have to be freshly isolated each time they are needed but have the advantage that they are differentiated and express their specialized functions. Cell culture systems are based on mechanical and/or enzymatic disaggregation of the tissue to single cells. Tissue samples are mostly obtained from slaughtered animals that are not killed for this purpose, so we can make the most of these animals.

Continuous cell lines show a growth pattern referred to as immortal. Although a few established cell lines are able to express specialized functions from the tissue or organ where they were derived, most are undifferentiated.

Mammalian and fish-derived cell lines have been frequently employed in short-term cytotoxicity assays. Extrapolation of in vitro toxicity results to the in vivo situation,

based on absolute toxicity values may differ significantly depending on the toxicant, however, good correlations have been determined based on the relative ranking order of pollutants to fish cells with their water-borne in vivo toxicity to live fish (Bols et al., 1985; Dierickx and van de Vyver, 1991; Segner and Lenz, 1993; Castaño et al., 1996).

There are more than 150 continuous cell lines that have been established from fish. Most of them are either fibroblast-like or epithelial-like, and originate mainly from the tissues of salmonids or cyprinids. Established fish lines were grown basically on basal culture media supplemented with mammalian sera, most of them were anchorage dependent and conventional tissue culture plastic ware was used for both culture and for testing (Fryer and Lannan, 1994). The major difference between mammalian and fish cell culture is the temperature. Most fish cell lines are unable to grow above 30 ºC and most are able to retain viability during long-term low temperature storage and grow normally after return to the optimum temperature.
In fish cell lines, growth and cellular functions are strongly temperature dependent. As a consequence, temperature affects the cytotoxic responses of fish cells (Babich and Borenfreund, 1987; Castaño et al., 2003; Fent, 2001; Plumb and Wolf, 1971; Wolf and Quimby, 1969).

RTG-2 cell line was the first permanent fish cell line established and was developed for studying fish viruses. In ecotoxicology, it has been mainly used for many years for the assessment of cytotoxicity. (Alañón et al., 2005; Riva et al., 2005; Babín et al., 2005; Sánchez-Fortún et al., 2005; Shúilleaháin et al, 2004; Alañón et al., 2003, 2001; Fent, 2001; Araujo et al., 2000; Lee, et al., 1988; López et al, 1998; Vega et al,.1995; Castaño et al., 1994a; 1994b; 1995a;1995b; 1996)

Bovine granulosa cells have been used in many studies too (Stacchezzini et al, 2006; Alañón et al, 2005; Vanholder et al., 2005; Tiemann et al., 1996; van Wezel et al., 1996, Langhout et al, 1991; Luck et al, 1990).

In the mature bovine ovary, as in other species (human, mouse), the vast majority of follicles are arrested at the primordial follicle stage, 94.3% of bovine follicles (Miyamura et al., 1996).

Each mammalian primordial follicle is typically composed of an immature and quiescent oocyte, completely and evenly surrounded by a single layer of flattened pre-granulosa

cells, with all enclosed by a basal lamina. Studies have shown that many ultrastructural features are consistent between species.
There was no zona pellucida present between the oocyte and the pre-granulosa cells in the quiescent primordial follicle of any species examined (van Wezel et al., 1996).

Follicular activation is the transition of a follicle from the (inactive) primordial follicle stage to the (active) primary follicle stage. At this time, the oocyte begins to enlarge and the granulosa cells begin to proliferate. Proliferation of cells in general, both in vivo and in vitro, entails a "rounding up" of cells prior to mitotic division (Alberts et al, 1989).

Likewise, the granulosa cells change in shape from flattened (primordial follicle) to cuboidal (primary follicle) at follicular activation. Additionally, upon follicle activation a zona pellucida is secreted between the growing oocyte and the granulosa cells, and is evident by the primary follicle stage in most of the species studied.
The main functions of granulosa cells include the production of esteroids and LH receptors.

1.4. In vivo methods

1.4.1 Vertebrates. The use of fishes

For several reasons, fish species have attracted considerable interest in studies assessing biological and biochemical responses to environmental contaminants (Powers, 1989).

Fish can be found virtually everywhere in the aquatic environment and they play a major ecological role in the aquatic food-webs because of their function as a carrier of energy from lower to higher trophic levels (Beyer and Sikoski, 1994).

The understandings of toxicant uptake, behavior and responses in fish have a high ecological relevance. But, there is also recognition of the need to avoid the unnecessary use of fish and other vertebrates in ecotoxicology. The published literature contains many proposals for incorporating the 3Rs (Reduction, Refinement, and Replacement) concept (Russell and Burch, 1959) into fish acute toxicity testing (Walker et al., 1998, 1997). These include new testing strategies to reduce the number of fish for acute tests (Jeram et a., 2005; Hutchinson et al., 2003; Douglas et al., 1986), the use of fish embryos (Canaria et al., 1999, Nagel, 2002 and Schulte and Nagel, 1994), in vitro assays based on fish cells or cell lines (Castaño et al., 2003), and QSAR strategies (Cronin et al., 2003 and European Commission, 2003b) to replace tests with free-feeding larvae, juvenile or adult fish.

The European Centre for the Validation of Alternative Methods (ECVAM) has recently established an Ecotoxicology Task Force to proactively identify further opportunities to improve the implementation of the 3Rs in ecotoxicity testing. One activity was the evaluation of the applicability of the fish acute threshold (step-down) test concept to new chemical substances using the approach described by Hutchinson et al. (2003) for human pharmaceuticals, which demonstrated a possible reduction of 73.2% in the number of fish used.

In the European Union, data requirements for the notification of new substances are listed in the annexes to the Council Directive 67/548/EEC on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging, and labelling of dangerous substances (European Commission, 1967).

The amount of data required increases according to the quantity of substance put on the market (European Commission, 1992). The "base set" data are stipulated for all substances for which the marketing quantity exceeds one tonne per year per manufacturer (Annex VII.A of the Directive). These requirements include acute toxicity for freshwater fish (LC50 96 h), acute toxicity for daphnids (EC50 48 h), and growth inhibition test on freshwater algae (growth rate: ErC50 72 h and/or biomass: EbC50 72h). After submission by industry, these "base set" data contained in summary notification dossiers are entered into the New Chemicals Database (NCD) hosted by the European Chemicals Bureau (ECB). The reported data are used for deciding on the classification and labelling as well as for hazard and risk assessment (calculation of Predicted No Effect Concentration—PNEC) of the substance.

The tests for a notification have to be carried out according to testing methods described in Annex V of the Directive 67/548/EEC (for fish, daphnids, and algae: methods C.1, C.2, and C.3 respectively; downloadable from the ECB website) and in compliance with the principles of Good Laboratory Practice. These methods are harmonised with corresponding test guidelines of the Organisation for Economic Cooperation and Development (Test Guidelines 201, 202, and 203; OECD, 1984, OECD, 1992 and OECD, 2004)

The current fish acute toxicity test (Annex V Testing Method C.1 or the corresponding OECD Test Guideline 203) determines the median lethal concentration for fish after 96 h of exposure to a range of concentrations of the test substance (LC50 96 h). It recommends the use of a minimum of seven fish per concentration and control. In practice, regulatory studies conducted by industry laboratories often use five exposure concentrations plus a dilution water control, giving a total of 42 fish per test. This number excludes any previous range finding test such as using five fish in a single concentration plus five control animals, where the concentration is usually selected on the basis of the physical–chemicals properties (e.g., log Kow or water solubility) of the testing substance, and previous testing of similar substances (read across). In fact, this range-finding approach offers a pragmatic basis for developing a fish acute threshold approach (Hutchinson et al., 2003).

1.4.2. "The use of microalgae"

It is very important to expand the knowledge about harmful effects of toxic substances on population growth of planktonic algae, since they are an important component of aquatic systems (Sáenz et al., 1997).

Planktonic algae are important organisms of the aquatic ecosystems, affected by chemicals, especially pesticides (Butler 1977). Freshwater microalgae have been used in numerous studies (Aguayo et al, 2000; Riva et al., 2001, 1994; 1993a, 1993b; 1992; Alañón et al., 2003; Gibert et al., 2002; Vallés, 2001, Lopéz, 1999; Moreno-Garrido et al., 2000; Bengtson et al., 2005, Ma et al., 2001a, b, 2002a, b, c, 2003, 2004, 2005). However, only few scientists had studied the ecotoxicity of chlorpyrifos and carbofuran on algae (Mukherjee et al., 2004; Kar and Singh 1978; Padhy 1985; Megharaj et al 1989; Antón et al 1993)

Algae play an important role in the equilibrium of aquatic ecosystems, being the first level of the trophic chain to produce organics and oxygen (Campanella et al., 2000). Furthermore, the tested species is often not a representative of the field communities of concern. Since response to toxicants can differ among algal species by 2–3 orders of magnitude, toxicity levels cannot simply be extrapolated from one species to others or to natural assemblages (Blanck et al., 1984; Kasai et al., 1993; Joern and Hoagland, 1996). Single-species laboratory studies may be inadequate for predicting the effects of chemicals on ecological communities.

A test organism's sensitivity to toxic substances is a complex issue. Some studies have shown that the sensitivities of plants and other groups of organisms vary widely among toxicants (Wang and Freemark, 1995). Some reports have been published about the comparative toxicity of solvents toward various test organisms (Tadros et al., 1994). A few reports involved the differential responses of various species to pesticides (Kasai et al., 1993; Ferrando et al., 1996).

1.4.3. The use of bacteria

The soil microorganisms, bacteria in particular, play an important role in the environmental fate of soil-applied pesticides. The ability of bacteria to metabolize pesticides and/or their metabolites for their benefit has been well documented (Sudhakar-Barik et al., 1979; Racke and Coats, 1987). One result of this catabolism is the failure of some pesticides to adequately control the target pests because of decreased persistence (Felsot, 1989). Determine the toxicities of pesticides to all possible soil bacteria involved in the enhanced degradation process would be very expensive and time consuming. The Microtox® system utilizes Vibrio fischeri, (previously known as Photobacterium phosporeum) a marine bacterium that is phylogenetically related to several genera of bacteria important in soil.
Bioluminiscence inhibition in the marine bacterium Vibrio fischeri is relevant to assessing a compound's impact on the microbial ecology. (Alañón et al, 2002; Riva et al., 2002; Fernández-Alba et al., 2001; Ribó et al., 1990, 1989, 1987; Ghosh et al., 1997).

The Microtox® system was originally developed to assess the toxic effects of complex industrial effluents (Bulich, 1984; 1981). Since its introduction in 1979, the application of the Microtox® system has been extended to determine the toxicity of aquatic pollutants, wastewaters, fossil-fuel process waters, mycotoxins, and numerous other chemicals (Ribó and Kaiser, 1987). Some state regulatory agencies employ the microtox system in screening tests to monitor environmental pollutants.

Nowadays, the Microtox® assay is employed in evaluating the toxicity of environmental samples and it is very used in order to assess the impact of pesticides used in agricultural production (Jones et al., 2003; Fernández-Alba et al., 2001; Ghosh et al., 1997; Ruiz et al., 1997; Somasundaram et al. 1990).
Microtox® measures the decrease in respiration, and subsequent light output, of the luminescent bacterium Vibrio fischeri as the toxic response. The Microtox® test is a simple, reliable test for screening microorganism toxicity. The test measures the fraction of luminescent bacteria that die in the test and the number of bacteria involved is measured in millions. Microtox® testing is a rapid, cost-effective tool in assessing toxicity of effluents, sediments, leachates, soils, sludges, groundwaters and surface waters. The EC50 of the Microtox® analysis is the sample concentration that reduces the reagent light output by 50%.

1.5 Biomarkers

One strategy to at least assess the contamination and its potential effects is the use of biomarkers in ecological surveys to verify the bioavailability and presence of active concentrations of pollutants in biota (Bucheli and Fent, 1996).

Van der Oost et al., 2003 in a review article explain that several definitions have been given for the term 'biomarker', which is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993).

According to Peakall, 1994 a biomarker is defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes), which can be related to exposure to or toxic effects of environmental chemicals.

Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response (NRC, 1987).

Van Gestel and Van Brummelen (1996) redefined the terms 'biomarker', 'bioindicator' and 'ecological indicator', linking them to different levels of biological organization.
They considered a biomarker as any biological response to an environmental chemical at the subindividual level, measured inside an organism or in its products (urine, faeces, hair, feathers, etc.), indicating a deviation from the normal status that cannot be detected in the intact organism. A bioindicator is defined as organism giving information on the environmental conditions of its habitat by its presence or absence or by its behavior, and an ecological indicator is an ecosystem parameter, describing the structure and functioning of ecosystems.

In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

According to the NRC (1987), WHO (1993), biomarkers can be subdivided into three classes:

Biomarkers of exposure: *covering the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism.*

Biomarkers of effect*: including measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease*

Biomarkers of susceptibility: *indicating the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure.*

In order to assess exposure to or effects of environmental pollutants on aquatic ecosystems, in the following list there are some of the suitable biomarkers (biological and biochemical parameters):

1. *Biotransformation enzymes*
2. *Oxidative stress parameters*
3. *Biotransformation products*
4. *Haematological parameters*
5. *Immunological parameters*
6. *Reproductive and endocrine parameters*
7. *Genotoxic parameters (Comet assay)*
8. *Neuromuscular parameters (Inhibition of Acetylcholinesterase)*
9. *Physiological, histological and morphological parameters*

1.5.1. Biotransformation enzymes

Generally, the most sensitive effect biomarkers are alterations in levels and activities of biotransformation enzymes. Enzyme induction is an increase in the amount or activity of these enzymes, or both.

Two major types of enzimes involved in xenobiotic transformations are distinguished:
Phase I enzymes; ethoxyresorufin O-deethylase (EROD), CYP1A
Phase II enzymes and cofactors: GST, UDPGT

1.5.2. Oxidative stress parameters

Many environmental contaminants (or their metabolites) have been shown to exert toxic effects related to oxidative stress (Winston and Di Giulio, 1991).

Oxygen toxicity is defined as injurious effects due to cytotoxic reactive oxygen species (ROS), also referred to as reactive oxygen intermediates (ROIs), oxygen free radicals or oxyradicals (Di Giulio et al., 1989). Of particular interest are the reduction products of molecular oxygen, which may react with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and, ultimately, cell death (Winston and Di Giulio, 1991). The activities of the antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which defend the organisms against ROS, are critically important in the detoxification of radicals to non-reactive molecules.

1.5.3. Biotransformation products

The stress proteins also called heat-shock proteins (HSP) comprise a set of abundant and inducible proteins involved in the protection and repair of the cell against stress and harmful conditions (Sanders, 1993). Special groups of stress proteins are the metallothioneins (MTs), which are inducible by both essential and toxic heavy metals (Stegeman et al., 1992; Viarengo et al., 2000), and the P-glycoproteins of the multixenobiotic resistance (MXR) mechanism, which may be induced or inhibited by a wide variety of chemicals (Bard, 2000).

1.5.4. Haematological parameters

Several haematological parameters are potential effect biomarkers. The leakage of specific enzymes (e.g. serum transaminases such as alanine transaminases (ALT), asapartate transaminases (AST)) into the blood may be indicative of the disruption of cellular membranes in certain organs. Although less specific, other haematological parameters, like hematocrit, hemoglobin, protein and glucose, may be sensitive to certain types of pollutants as well. In addition, the blood levels of specific steroid hormones or proteins normally induced by these hormones may be indicative for certain reproductive effects due to endocrine disruption.

On the other hand, blood samples can regularly be obtained from test organisms, thus allowing the use of a non-destructive approach in effect assessment and reducing the use of test animals.

1.5.5. Immunological parameters

The immune system comprises a network of cells capable of rapid proliferation and differentiation, regulated by a variety of soluble factors, and is closely integrated with other organ systems and functions. As such, it is extremely vulnerable to exogenous chemicals, especially after chronic exposure or repeated short exposures (Weeks et al., 1992). In mammals it was observed that both cell-mediated and humoral (antibody-mediated) immunity may be depressed by pollutants such as PAHs, PCBs, PBBs, OCPs (e.g. dieldrin, lindane, DDTs and HCB), organometals (e.g. methylmercury and organotins) and heavy metals such as Pb and Cd (Vos et al., 1989). It should, however, be emphasized that the immune system can be influenced by a large variety of stressors, which implies that immunological biomarkers may be useful and sensitive, but often nonspecific (reviewed by Weeks et al., 1992).

1.5.6. Reproductive and endocrine parameters

The impact of xenobiotic compounds on reproductive and endocrine effects has attracted growing interest in recent years. Hormone regulation may be impaired as a consequence of exposure to environmental pollutants (Spies et al., 1990).
A number of xenobiotics with widespread distribution in the environment are reported to have endocrine activity, which might affect reproduction and thus might threaten the existence of susceptible species (Colborn et al., 1993; Peterson et al., 1993; White et al., 1994). Animals at high trophic levels, generally having limited reproduction rates, are likely to be the most vulnerable in this regard. An example is the occurrence of imposex (development of male sexual characteristics in female gastropods) in the Gastropod Bolinus brandaris due to organotin exposure

The synthesis of VTG (vitellogenin), a precursor of yolk proteins, is affected by estradiol. It was demonstrated that PCB exposed fish were less capable of producing VTG (Spies et al., 1990). An impaired reproductive function due to decreased plasma VTG levels was reported for female rainbow trout exposed to Cadnium (Haux et al., 1988). VTG synthesis can also be induced in male fish exposed to endocrine disrupting chemicals such as alkylphenols, thus leading to a so-called feminisation of male fish (Gimeno et al., 1996). The VTG response in fish has been used as a sensitive biomarker of exposure to estrogenic compounds in many studies (García-Reyero et al., 2004; Lavado et al., 2004; Solé et al., 2003a; 2003b)

Another potential biomarker for estrogenic effects in male fish might be the induction of zona radiata proteins (ZRP), also known as vitelline envelope proteins (Hyllner et al., 1991). Recent studies with juvenile salmon indicated that the ZRP response was more sensitive to various environmental pollutants than the VTG response (Arukwe and Goksøyr, 1997; Arukwe et al., 2000), thus providing a sensitive means of detecting exposure to environmental estrogens. Both VTG and ZRP can be analyzed by measuring mRNA expression or plasma protein levels.

1.5.7. Genotoxic parameters

Many pollutants released into the aquatic environment have the capability to damage the genetic material of exposed organisms. As a result, genetic disorders, diseases, and mortality may occur. Several epidemiological studies have demonstrated correlations between the genotoxic of the aquatic environment and carcinogenic effects in wildlife as well as in humans e.g., Griffith and Riggan, 1989; Black and Bauman, 1991; Anderson and Wild, 1994.

The exposure of an organism to genotoxic chemicals may induce a cascade of events (Shugart, 1992): formation of structural alterations in DNA, procession of DNA damage and subsequent expression in mutant gene products, and diseases (e.g. cancer) resulting from the genetic damage. The detection and quantification of various events in this sequence may be employed as biomarkers of exposure and effects in organisms exposed to genotoxic substances in the environment. DNA modifications, such as strand breaks, are biomarkers for genotoxic chemicals, for example COMET assay and DNA unwinding assay.

1.5.7.1. Comet assay

The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows the assessment of the DNA damage.

The alkaline version of this method, as developed by Singh et al., 1988, detects DNA single and double strand breaks, alkali-labile sites, and incomplete excision repair events in single cells. Alkaline treatment of cells followed by electrophoresis at high pH results in streaming of damaged DNA toward the anode, giving the appearance-after DNA visualization by fluorescent dyes-of the tail of a comet. The tail length, the DNA amount in the tail or the product of these two parameters-the so-called tail moment-are the measures to quantify the extent of DNA damage (Ashby et al., 1995).
The comet assay, which has been originally developed for application with mammalian cells, recently has been successfully adapted to be used with fish cells in vitro

(Schnurstein et al. 2001; Nehls and Segner, 2001; Devaux et al., 1997), which have increased the utility of this assay for environmental surveillance and monitoring.
Fish cell lines have been repeatedly chosen for in vitro genotoxicity screening of chemicals, chemical mixtures, and samples from the aquatic environment for reviews see Segner, 1998; Tarazona et al., 1993; Babich and Borenfreund, 1991).

The use of fish cells in aquatic genotoxicity assessment is based, in part, on the observation that they retain some fish specific traits, for instance, the poikilothermic nature, peculiarities of xenobiotic metabolism, or low rates of DNA repair (Kocan et al., 1985; Shugart, 1995).

But for fish cell lines, few data are available on their metabolic capabilities and related genotoxic reaction patterns (Thornton et al., 1982; Walton et al., 1984; Smolarek et al., 1987).

1.5.8. Neuromuscular parameters

With respect to neural functions, enzymes of interest are cholinesterases (ChE) (Payne et al., 1996). The inhibiton of the activity of ChEs has been frequently used as a biomarker to assess the extent exposure to organophosphorous and carbamates, principally in vertebrate species (Walker and Thomson, 1991).
Organophosphorous and carbamates pesticides are known to inhibit esterases, by binding to the active site and phosphorylating the enzyme.

The interaction of Ops with some esterases led to the classification of these enzymes into the three categories:

- **A-esterases**, *which are able to hydrolyze OPs and are not inhibited by them.*
- **B-esterases-cholinesterases** *(ChEs) and carboxylases (CaEs), which are typically inhibited by OPs*
- **C-esterases**, *which no interact with this family of pesticides (Aldridge and Reiner. 1972; Parkinson 1996)*

Two types of ChEs are recognized; firstly, those with a high affinity for acetylcholine (AChE, EC 3.1.1.7), and secondly, those with affinity for butyrylcholine (BChE), also known as non-specific esterases or pseudocholinesterases (Walker and Thompson, 1991; Sturm et al., 2000).

AChE is involved in the deactivation of acetylcholine at nerve endings (Figure 5), preventing continuous nerve firings, which is vital for normal functioning of sensory and neuromuscular systems (e.g. Murphy, 1986). It is, therefore, assumed that this enzyme is more important than the non-specific esterases. This assumption, however, can only be confirmed when the physiological functions of non-specific esterases are elucidated more fully.

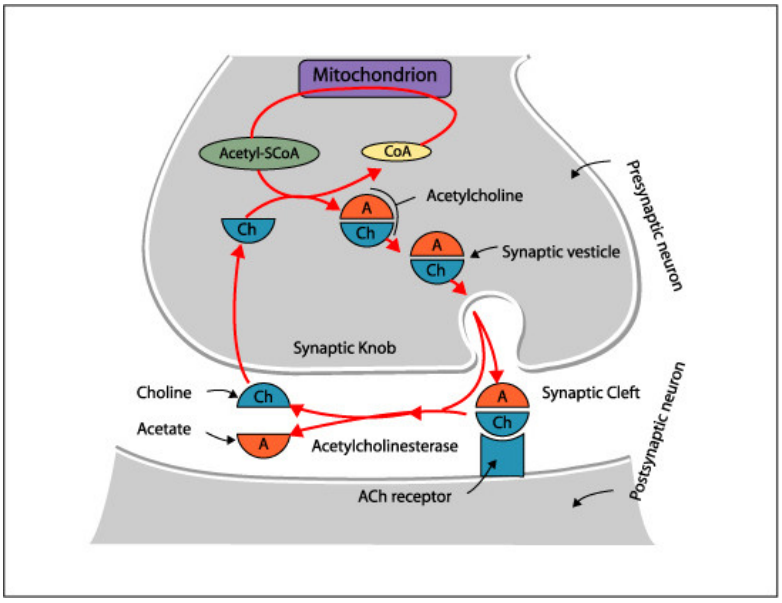


Figure 5. Action 's mechanism of the enzyme Acetylcholinesterase

1.5.8.1. Inhibition of Acetylcholinesterase (AChE)

Many organophosphate (OP) and carbamate (C) pesticides are reported to be effective AChE inhibitors. They disrupt transmission in the central and peripheral cholinergic nervous systems in vertebrates by inhibiting acetylcholinesterase activity (Sahib and Rao, 1980; Sharma et al., 1993).

The OP pesticides are acutely neurotoxic. They are designed to be effective inhibitors of the enzyme acetylcholinesterase located at neuromuscular junctions in the central and peripheral nervous system (Walker et al., 2001). The AChE inhibition has, therefore, been used to assess the nature and extent of the exposure of wildlife to agricultural and forest sprays (e.g. Greig-Smith, 1991; Zinkl et al., 1991).

OPs and carbamates may pose risks to non-target organisms (Basack, et al., 1998). Although the initial success of these pesticides was mainly founded on their high biological specificity and rapid environmental degradation, their high biochemical specificity has resulted in the development of resistance in many insect and mite pests

(Roush and McKenzie, 1987; Taylor and Feyereisen, 1996). Furthermore, most OPs and carbamates are also toxic to nontarget organisms (Da Silva et al., 1993; Detra and Collins, 1991) and some appear to have relatively high environmental persistence (Eto, 1974). Thus, in the same manner as resistance development among target pests, populations of nontarget organisms living near areas sprayed with OPs or carbamates could become adapted by developing increased resistance (Barata et al., 2001).
The persistent inhibition of acetylcholinesterases causes neurotoxic effects (O'Brien, 1960). In mammals, death is caused by asphysxia (Murphy, 1986), but the action in aquatic organisms including fish and invertebrates is less clear since as long as water crosses respiratory organs (i.e. gills), transfer of oxygen occurs (Day and Scott, 1990; Boone and Chambers, 1996).

A number of studies have successfully used ChE inhibition in fish as a biomarker of pesticide exposure (Bretaud et al., 2000, Dembélé et al., 2000; Chuiko, 2000; de la Torre et al., 2002, Shaonan et al., 2004; Küster, 2005; Senger et al., 2005; Rendón-von Osten et al., 2005; Selvi et al., 2005). There are several published studies on the use of ChE measurements as exposure biomarkers on cells (Bartos et al., 1976; Ott et al., 1982; Szelenyi et al., 1982; Paulus et al., 1981; Jin QH, 2004, Marinovich et al., 1996). Evidence shows that ChEs are polymorpphic enzymes, which may be soluble or bound to membranes depending on species and tissues (Basack et al., 1998).

The role of acetylcholinesterase in terminating acetylcholine-mediated neurotransmission made it the focus of intense research for much of the past century. But the complexity of acetylcholinesterase gene regulation and recent evidence for some of the long-suspected 'non-classical' actions of this enzyme have more recently driven a profound revolution in acetylcholinesterase research. Soreq and Seidman, 2001 summarize the evidences on a remarkable diversity of acetylcholinesterase functions.

As stated above, acetylcholinesterase (AChE) is responsible for inactivating acetylcholine at cholinergic synapses in both the central and peripheral nervous systems (Figure 5). AChE is classified into R, H, and T transcripts depending on the structure of the encoded C-terminal domain, formed from alternative splicing of a single gene in vertebrate (Massoulie and Bon, 1982).
However, the enzyme is not restricted to inactivate acetylcholine and accumulated evidence increasingly points to non-catalytic roles (Soreq and Seidman, 2001)

AChE also exists in WRL-10A cultured fibroblasts a subline of L-929 mouse fibroblasts (Bartos et al., 1976), human erythrocytes (Ott et al., 1982), T lymphocytes (Szelenyi et al., 1982) and murine thrombocytic cells (Paulus et al., 1981), , also Jin QH, 2004 studied the potential function of acetylcholinesterase (AChE) in Normal Rat Kidney (NRK) cells. In all these cells, the enzyme has no obvious relations to its classic function.

It is well established that neurotransmitters, mainly catecholamines and neuropeptides, have specific roles in the regulation of ovarian functions, including modulation of steroidogenesis, gene induction, and initiation of follicular growth. These neurotransmitters are derived from the ovarian sympathetic innervation and/or from intraovarian sources. Ovarian tissue, however, binds cholinergic agents and may thus contain ACh-receptors. Studies with cultured ovarian endocrine cells, namely granulosa cells suggested functionality of these receptors. Luck, 1990 reported cholinergic stimulation of steroid production of bovine granulosa cells.

Since ACh is a known neurotransmitter, ovarian nerves appear in theory to be obvious potential sources of ACh. Ovarian nerves release neurotransmitters by forming synapses "en passent" while they travel with blood vessels through endocrine interstitial cells, the theca cell layer of follicles and possibly through the corpus luteum (CL). It is important to mention that granulosa cells are not in contact with nerve fibers. Nerve fibers do not cross the border formed by the basal lamina, which separates thecal cells from granulosa cells of follicles. Therefore, if contained and released from ovarian nerves, ACh would have to reach granulosa cells by diffusion. However, ACh is a labile, short-lived molecule, which is rapidly degraded by cholinesterases contained in serum and in the ovarian interstitial and follicular fluid. It is therefore unclear whether ACh derived from nerve fibers could reach granulosa cells and activate ACh receptors

In a paper by Burden and Lawrence, 1978 a histochemical staining procedure indicated that typical sympathetic ovarian nerve fibers also possess AChE activity. The results were taken as indication of the presence of the neurotransmitter ACh in sympathetic ovarian nerve fibers. Instead based on the assumption by Burden and Lawrence, 1978 who suggested that some fibers from the vagus nerve may travel along with sympathetic fibers to the ovary, a cholinergic innervation pattern of the ovary similar to the one of catecholaminergic nerves was assumed.

Granulosa cells of growing follicles not only produce hormones, but are also proliferating cells.

In embryonic tissues, neurons as an obvious source for ACh do not (yet) exist. Therefore non-neuronal cells provide the only possible sources of ACh. This situation in the embryo is reminiscent of various tissues in the adult, which remain devoid of innervation, but possess functional receptors for ACh. Several of these tissues, including immune cells, epithelial and endothelial cells, as well as placental cells (Kawashima et al., 2000; Anesseti et al., 2001) have only recently been recognized to be able to produce ACh. These cells, which are non-neuronal in nature indicate that "cholinergic systems", in which non-neuronal ACh may exert regulatory influences on producers and neighboring cells, exist throughout the body.

There are two families of receptors for ACh. Nicotinergic ACh receptors are ligand-gated ion channels, while muscarinic ACh receptor (MR) types are G-protein coupled receptors. While the possible existence of nicotinergic ACh receptors in the ovary remains to be examined, evidence for ovarian MRs was provided by Batra et al, 1993 and Arellano et al., 1999. Mayerhofer et al., 2003 revealed MR of M1, M3 and M5 types in the human and nonhuman primate ovary using RT-PCR and immunohistochemistry.

These form a subgroup, which is linked to activation of phospholipase C and protein kinase C, as well as to Ca2 +-release from intracellular stores. RT–PCR and immunohistochemical results indicated that M3 is present in oocytes, while M1 and M5 genes were expressed in granulosa cells. The results of Mayerhofer suggested that ACh produced by follicular and luteinizing granulosa cells may act in auto-/paracrine ways within the ovary on oocytes and/or on granulosa cells /luteal cells. Importantly, the sites of production and the sites of action are in either case in immediate proximity, thereby allowing the short-lived molecule ACh to exert its action immediately after release and prior to the presumed degradation by esterases contained in follicular fluid.

Single cell Ca^{2} + measurements of granulosa cells loaded with FURA 2 (as calcium indicator) showed that M1/M5 are functional. ACh analogs (eg. carbachol or the MR agonist oxotremorine M) increased free intracellular Ca^{2} +-levels within seconds.

Similar effects of cholinergic agents were reported in chicken granulosa cells. In human granulosa cells, M1-selective antagonist pirenzepine prevented intracellular Ca^{2} +-

elevation. Furthermore, sustained M1/5 activation by carbachol for 24 hours stimulated granulosa cells proliferation in vitro.

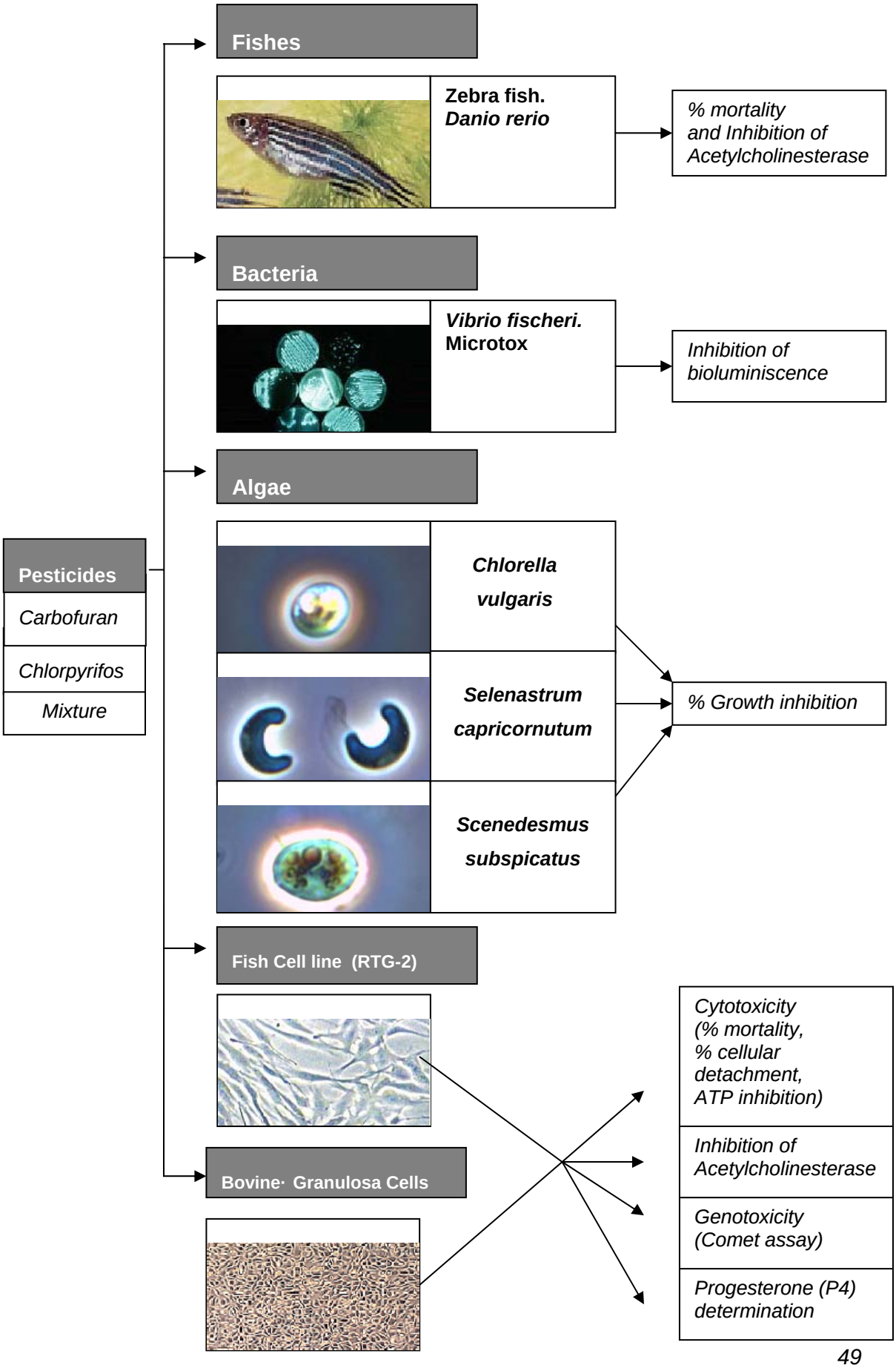
Fritz et al., 1999 reported that ACh can act as a trophic factor in granulosa cells. However, how M1/5 receptor activation is linked to proliferation is currently unknown. However, proliferation appears to be negatively correlated with gap junctional communication in granulosa cells.

1.5.9. Physiological, histological and morphological parameters

These parameters are higher-level responses following chemical and cellular interaction, which are generally indicating of irreversible damage (Hinton et al., 1992). Determination of adverse effects can be performed histopathologically, by investigating lesions (Vallés, 2001), alterations or tumour formation (neoplasms) in tissues.

Experimental Design

2 EXPERIMENTAL DESIGN



2.1. Objectives

Toxicity data from laboratory tests with single pure chemicals provide an essential input to scientific assessments of chemical risks to aquatic life. Aquatic organisms, however, are rarely exposed to only one single contaminant, but typically to mixtures of numerous man-made-chemicals with varying constituents in varying concentrations and concentration ratios. The direct testing of a lot of potential combinations of water contaminants is unfeasible, and thus we are confronted with the task of deriving valid predictions of mixture toxicity from toxicity data on individual compounds.

Pesticides found in the aquatic environment usually occur as mixtures, not as single contaminants (Gilliom et al., 1999). Scientific assessments of chemical risks to aquatic life have to deal with this complex exposure situation, but rely on toxicological data as a critical input. Unfortunately, the vast majority of available toxicity data relates to single pure substances, not to mixtures.

The response of a single toxicity assay is an insufficient measure of the adverse biological impact of a compound in a generally diverse receiving ecosystem. Different toxicants act differently and not all life forms are equally susceptible. Consequently, single bioassays will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be used (Bierkens et al., 1998, Isooma et al., 1995). A minimum ecotoxicological in vitro test battery should at least include bacteria, protozoans, algae, invertebrates fish cell lines, or cells isolated from fish and other species.

The application of prokaryotic tests systems with biomarkers such as AChE activity and DNA fragmentation in different tissues of test organisms seems to be a useful combination for the assessment of cytotoxic and genotoxic potential in environment.

The aims of the present study are:

- *Demonstrate the utility of two different in vitro system represented by a continous fish cell line and primary culture of mammalian cells, to obtain information about the toxicity of carbofuran and chlorpyrifos and to evaluate to what degree the toxic effect of pesticides is modified when mixed.*

- *Compare differences between established cell lines and primary cells as well as to compare fish to mammal's responses.*

- *Contribute to the development of alternative methods to the use of laboratory animals comparing the in vitro responses to in vivo zebra fish results.*

Materials and Methods

3. Materials and Methods

3.1. Pesticides

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Chlorpyrifos (0,0-diethyl-0-(3,5,6-trichloro-2-pyridyl)-phosphorothioate)) and carbofuran (2,3-dihydro-2,2-dimethyl-7 benzofuranol methylcarbamate)) were purchased from Sigma (St Louis, MO). The purity range was 99.2 and 99.9% respectively.

Solutions were made into standard stock solutions by dissolving in ethanol. The stock solutions were after diluted to obtain a range of different concentrations of exposure solutions in different media according to each experimental. The exposure solutions were added to the systems, and incubated for the adequate exposure time period.

Pesticide mixtures were produced by combining various stock solutions of pure components. When using pesticide mixtures, the concentration of the toxicant was defined as the sum of concentrations of all the pesticides in the test vial or solution. Synergistic and antagonistic toxicity effects were observed with pesticide cocktails relative to pure compound toxicities
The combined effects of insecticides were determined as follows: the two insecticides being tested were added at the same concentration.

3.1.1. Concepts for predicting multiple mixture toxicity

Numerous methods and models for analysis and assessment of combined effects of chemical compounds have been introduced in different fields of pharmacology and toxicology. In essence, however, most of them are based on only two different fundamental concepts (Boedeker et al., 1992). Commonly these are called concentration addition and independent action, but both concepts have also been used under various other names. In particular, response addition is often used as a synonym for independent action. A short introduction to these concepts was given by Könemann and Pieters (1996). Extensive discussions from differing viewpoints and guidance to the extensive literature on these concepts can be found in Pöch (1993) and Greco et al. (1995).

Concentration addition and independent action represent different hypotheses on a functional relationship between the toxicity of single substances and those of combined toxicants. In case that the components of a chemical mixture share a common mechanism of action, the concept of concentration addition is generally accepted as a reasonable expectation for their joint toxicity (Calamari and Vighi, 1992). Supporting evidence comes from multi-component mixture toxicity studies with different groups of toxicants and different types of aquatic organisms (Barata et al., 2006; Altenburger et al., 2000; Xu and Nirmalakhandan 1998). In the opposite case of chemicals with different modes and mechanisms of action the predictability of their combined toxicity and the choice of the most appropriate concept for prediction is a highly controversial issue, due to lack of conclusive theoretical arguments and experimental evidence.

3.1.2. Calculating concentration addition

The concept of concentration addition can be traced back to the early work of Frei (1913) and Loewe and Muischnek (1926).

Usually it is defined for a binary mixture of substances 1 and 2 by the equation

$$\frac{c_1}{\mathrm{EC}x_1} + \frac{c_2}{\mathrm{EC}x_2} = 1. \quad (1)$$

but can be extended to any number of n components (Berenbaum, 1985) by

$$\sum_{i=1}^{n} \frac{c_i}{\mathrm{EC}x_i} = 1. \quad (2)$$

In this equation, c_i are the concentrations of the individual substances present in a mixture with a total effect of x%. ECxi are the equivalent effect concentrations of the single substances, i.e. those concentrations that alone would cause the same quantitative effect x as the mixture. Quotients ci /ECxi express the concentrations of mixture components as fractions of equi-effective individual concentrations and have been termed toxic units (Sprague, 1970). If Eq. (2) holds true, a mixture component can be replaced totally or in part by an equal fraction of an equi-effective concentration of another without altering the overall effect of the mixture. Or in other words, the total effect of the mixture is expected to remain constant as long as the sum of the toxic units remains constant.

Concentration addition is based on the idea of a similar action of mixture components. In the case that chemicals meet this requirement of similar action, concentration addition is generally accepted as a reasonable expectation for their joint toxicity (Calamari and Vighi, 1992). However, interpretations of the term similar action differ considerably. From a phenomenological point of view the term may be used in a very broad sense, comprising all substances which are able to cause the toxicological response under consideration. If we are dealing with integral endpoints, like death of an organism or inhibition of population growth, this may apply to almost all substances, only dependent on bioavailable concentrations. Indeed concentration addition has been suggested as the 'general solution' to the problem of calculating an expectable effect for a combination of agents (Berenbaum, 1985). On the other hand, from a mechanistic point of view, similar action may be understood in a very strict sense, and regarded to be relevant only in the special case of competitive and reversible interaction of specifically acting toxicants at an identical molecular binding site (Pöch, 1993). With this perspective, not even the existence of a common specific target site is a guarantee for concentration additive mixture toxicity on the organism level of response. Additional binding to different unspecific sites or dissimilar toxicokinetic characteristics of chemicals may result in an altered mode of combined action.

The alternative concept of independent action (or response addition) was first formulated by Bliss (1939). In contrast to concentration addition, independent action is based on the idea of a dissimilar action of mixture components. Dissimilar means the primary interaction of toxicants with different molecular target sites and the triggering of a common toxicological endpoint via distinct chains of reactions within an organism.

3.2."In vitro assays

3.2.1. Fish cell line RTG-2

RTG-2 fibroblastic cell line (ATCC nº55) (Figure 6) was established by Wolf and Quimby (1962) from rainbow trout gonads (Oncorhychus mykiss). The cells were kindly provided by Dra. Argelia Castaño, CISA-INIA, Madrid.

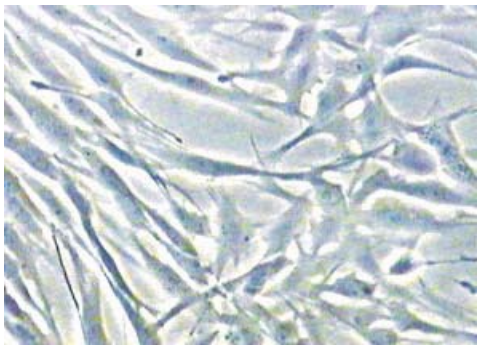

Figure 6. Monolayer of RTG-2 cell line after 48 h of culture. Contrast phase 40x.

3.2.1.1. Cell culture

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Cells were grown on 75 cm^2 tissue culture flasks (Costar), in Eagle's Minimum Essential Medium (MEM), with non-essential amino acids, 200 IU penicillin/ml, 100 μg streptomycin/mL and 0.85 g sodium bicarbonate/L, supplemented with 1.25 μg amphotericine/L, 2 mM glutamine and 10 % foetal calf serum (FCS) at 20ºC+/-1ºC in a 5% CO2 atmosphere in a Heareus incubator (more details in annex 1 of material and methods in Table 1).

For subcultivation, the cells were detached using an EDTA/trypsin solution (1mM EDTA/0.25% trypsin) in Ca2+ - and Mg2+-free Hanks Balanced Salts Solution (HBSS).

3.2.1.2. Exposure conditions

Cells were seeded in 96-well culture microtiter plates at $2x10^4$ cells/well and incubated for 48 h at 20ºC. After this acclimation period in which cells reached exponential growth-phase, the culture medium was removed and replaced by the test medium (samples + medium containing 1% instead 10% of foetal calf serum).

This seeding density was found to be optimal to achieve the desired confluency (60–70% following 48 h in culture). In order to avoid the effects of medium evaporation from the outer wells of the microplate, only the internal 48 wells were employed for cytotoxicity testing.

Fish cells were exposed to chlorpyrifos, carbofuran and the mixture. Untreated control wells received medium without test agents. A solvent control, ethanol at a final concentration of 2% (v/v), was included in all assays. Six replicate wells were used for each control and test concentration per microplate. Pesticide exposure was conducted for 48 h. After this period, morphological damage was evaluated in comparison to the controls. The test medium was removed; cell monolayers washed with phosphate buffered saline (PBS) and prepared for the cytotoxicity assays.

3.2.2. Bovine granulosa cells

3.2.2.1. Granulosa cell collection

Bovine ovaries (Figure 7) were collected at the local commercial abattoir of Sabadell from cows assessed visually as being nonpregnant. As soon as they were taken from the carcasses of the animals, they are washed in saline solution, phospate buffer solution (PBS) and immediately transported to the laboratory in plastic bags at 30ºC.

Figure 7. Bovine ovaries

The ovaries are then divided in several groups to create different cellular pools; each group of ovaries are washed three times with 0.9% saline solution in 1L beaker, then immersed in 70% ethanol for 30 s, and rinsed three times again with saline solution. The ovaries are kept in saline complemented with penicillin until the granulosa cells are collected.

The choice of the follicles (Figue 8) to aspirate were based on the surface diameter that should be less than 2-5 mm. Follicular fluid was repeatedly aspirated using a needle (20 gauge; 38.1 mm) and sterile syringe to disrupt the granulosa cell layer and were collected in 12 ml sterile tubes as described by others (Vanholder et al., 2005; Basini et al., 2002; Bosc and Nicolle, 1997; Spicer and Stewart, 1996).

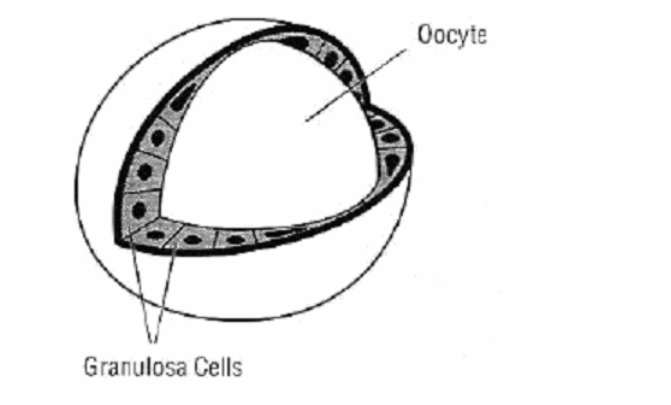

Figure 8. Bovine primordial follicle with a segment of the granulosa cells removed to show the arrangement of the granulosa cells relative to the oocyte.

The granulosa cells (Figure 9) are recovered by centrifugation at 2500rpm for 10 min and are washed three times with 7 ml of serum-free medium; at each wash, cells are separated from medium via centrifugation (2500 rpm at 4°C for 4-5 min). After the centrifugation the supernatant is aspirated with a vacuum and replaced with other 7 ml of M1 (see composition in annex 1 of material and methods in table 1); to disperse cell clusters the cell aggregate are resuspended several time through a Pasteur pipette.
Numbers of viable cells are determined using trypan blue exclusion method. 500 µl of M1, 20 µl of the final cell suspension and 100 µl of trypan blue are added togheter in 12 x 75 mm sterile tube. To count the percentage of viable cells 10 µl of the cell suspension-trypan blue solution were put in the hemacythometer and observed with a scope at 40x in bright field. If the cells are not stained with trypan-blue they were regarded as viable cells.

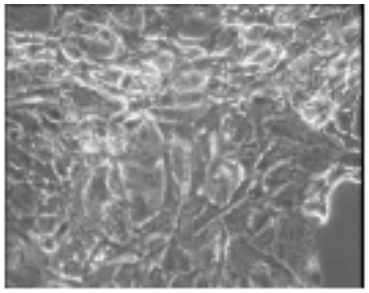

Figure 9. Monolayer of cells from bovine granulosa after 48h of culture. Contrast phase 40x

3.2.2.2. Granulosa cell culture

Aproximately 3 x10^5 cells/ml are added to 96 multiwell plates containing 200 μl of Eagle's Minimum Essential Medium (MEM), with non-essential amino acids, 200 IU penicillin/ml, 100 μg streptomycin/mL and 0.85 g sodium bicarbonate/L, supplemented with 1.25 μg amphotericineB/L, 2 mM glutamine and 10 % foetal calf serum (FCS) (more details in annex 1 of material and methods in Table 1).

Cultures were kept at 38.5° C+/-1ºC in a humidified 95% air-5% CO2 environment in a Jouan incubator. To obtain an optimal attachment, cells were maintained in the presence of 10% FBS (Foetal bovine serum) for the first 48 h of culture, unless stated otherwise. After this time, granulosa cells were washed twice with 0.2 ml of M3 (see composition in annex 1 of material and methods in Table 1) and incubations continued in M3 with or without added pesticides. During the first two days of culture the confluency of the cells were observed with a scope at 40x in bright field. We registered an increasing percentage of the cell confluency and changeovers in the cells' shape. An increased attachment on the bottom of the well can be noticed by an apparent increasing of the cell size and by the presence of simil-fibroblastic filaments which number and lenght increase during the days of culture. The size of the cells increases just apparently because the volume of the cells doesn't change; this can be explained considering that the "bigger" cells are plated down on the bottom of the well, and the "smallers" are still flotting in the medium.

3.2.2.3. Exposure conditions

Fish cells were exposed to chlorpyrifos, carbofuran and the mixture.

Six replicate wells were used for each control and test concentration per microplate. Pesticide exposure was conducted for 48 h. After this incubation period, morphological damage was evaluated in comparison to the controls. The test medium was removed, cell monolayers washed with phosphate buffered saline (PBS) and prepared for the cytotoxicity assays.

3.2.3. Cytotoxicity assays

Different endpoints have been used to assess acute cytotoxicity (Babich et al., 1986, Bols et al., 1985, Marion and Denizeau 1983, Parkinson and Agius, 1988). Some authors have compared the usefulness of each one, with the final purpose of choosing just one: the best of them (Halder and Ahne, 1990). On the other hand, other authors like Castaño, 1996 point to the convenience of using more than one endpoint in routine test by combining three well established assays in the simplest way, and on the same cell microplate, This point has special relevance for chemicals with unknown mechanisms of action, which can produce unrealistic results in certain endpoints.

Cytotoxicity was determined in RTG-2 cell line and bovine granulosa cells by measuring four endpoints, after 48 hr sample exposure: highest tolerated dose (HTD) (Liebsch and Spielmann, 1995), neutral red stain uptake (NRU) to evaluate cell viability (Borenfreund and Puerner 1985; Maracine and Segner, 1998), FRAME kenacid blue protein assay (KB) to evaluate cell detachment to the substratum (Knox et al. 1986) and intracellular ATP determination as an estimator of the energetic cell status (Castaño et al., 1994a, Kemp et al., 1986).

3.2.3.1. Highest tolerated dose (HTD)

The first endpoint examined cell morphology following exposure by determination of the HTD (Liebsch and Spielmann, 1995). The HTD is defined as the highest test substance concentration that causes the minimum morphological changes to cells. Following 48 h exposure to the toxicants, discernible changes observed in test wells such as alterations in cell shape, monolayer cell density and disruption of cell to cell contact, when compared to the control wells, were recorded using an inverted microscope (Nikon Eclipse TS-100) to establish the HTD.

3.2.3.2 Neutral red uptake and kenacid blue assays

Viability and protein determination of the cells following 48 h exposure to the pesticides were subsequently investigated using the NR and KBP assays. The incorporation of the NR dye by the lysosomes of living cells and the quantification of the total amount of cellular proteins were performed (Liebsch and Spielmann, 1995, Borenfreund and Puerner 1985; Maracine and Segner, 1998).

The Neutral Red Assay is based on the fact that one immediate effect of many toxics substances is damage to the cell membrane, which manifest as the leaking out of intracellular contents. If cell in culture are preloaded with vital dye and then exposed to various toxic concentrations, the amount of dye released from the cells will indicate the degree of membrane damage that has been caused and thus provide a measure of toxicity. The neutral red is preferentially taken into the lysosomes of the living cells.

The assay was performed as described by Borenfreund and Puerner (1985) but washing with PBS and adding only one half of the described amount of neutral red. Briefly, a stock solution of neutral red of 0.4% was prepared. 1mL of this stock was added to 100 mL of Minimum Essential Medium (MEM). To avoid the drawback of precipitation of neutral red dye into visible, fine, needle-like crystals, this solution was incubated 24 h before the assay at 37ºC and then centrifugated 15 min at 3500 rpm in order to induce this precipitation and eliminate the NR crystals which may interfere with the assay. 100 uL were added in each well of the 96-well microplate.

Following 3 h incubation to allow NR uptake, medium was eliminated by inverting carefully the plate on a filter sheet, cells were fixed with 200 μL/well of phormol-calcium soution (10% phormol, 10% calcium clorure and 80% distilled water) for 2 min. Then, the dye was extracted with an acetic acid–ethanol solution (1% acetic acid, 50% ethanol and 49% distilled water) (200 μL/well). After 15 min of agitation the absorbance of the solution in each well was measured at 550 nm with a Biotek 312E microplate reader spectrophotometer.

It is possible to perform the Kenacid Blue protein assay after the Neutral Red assay in the same plate because the cells fixed, protein determination or detachment of grown substrate was evaluate using the Kenacid Blue protein assay described by Knox et al. (1986) but adapted to use 96 well plates adding for 30 min 100 μL kenacid blue stain solution on the same plates immediately following NR determination.

One of the drawbacks of this assay is that the dye may precipitate out. The likelihood of this occuring increases as the length of handling time increase; therefore 96-well plates should be agitated and inspected visually for uneven blue colour. For this reason, in order to avoid the precipitation the plates were incubated in agitation during all the 30 min.

Three washes of the unbond stain were done adding 100 uL of washing solution the first time and 200 μL the next (Ethanol 10%, glacial acetic acid 5% and distilled water 85%). Washing solution was eliminated by suction with a vacuum pump. The extraction

of the dye was also modified. Only 50 μL of the desorb solution (1M potassium acetate in 70% ethanol) were initially added. Plates were kept in an ultrasonic bath for 1-2 minutes and then completed up to 200 μL of the desorb solution, sacked and read in a plate reader at 570 nm. In the conditions of the test the measure of protein content must be interpreted as cell detachment, because there is no cell population growth when FBS percentage is reduced to 1% (Lee and Bols, 1988).

3.2.3.3. ATP determination

The ATP assay was performed following the procedure described in the annex 2 of material and methods.

Briefly, the test medium from two wells of each concetration, including control wells, was removed carefully by suction, and cell were washed two times with 200 μL/well of PBS pH=4.

Then, 100uL of ATP Assay Mix solution were added to a reaction vial. It was swirled and allowed to stand at room temperature for approximately 3 minutes. During this period any endogenous ATP was hydrolyzed, thereby decreasing the background.

To a separate vial containing 100μL of Somatic Cell ATP Releasing Reagent, we added 50μL of phospate buffer (100 mM pH 7.4) and 50μL of the cell sample.

Then, 100μL was transfered to the reaction vial and immediately measured the emitted amount of light using a Sirius luminometer.

Values were transformed in ATP ng by means of daily-performed standard curves.

3.2.3.4. Cytotoxicity index

Values of each assay were presented as percentage of control values. In ordert to integrate the three endpoints in a single data Castaño et al., 1994b, established a cytotoxicity index using the published equation:

$$CI50=[(KBP50+0.72)*(NR50+0.71)*(ATP50)]/2.43$$

The index was applied to the set of data obtained for each concentration, so that an additional dose response curve for each chemical was obtained. NR50, KB50, ATP50 and Cl50 and its 95% confidence limits were calculated by computerized log-probit analysis using the SPSS© 11.5 package

3.2.3.5. Statistics

Absolute values of each assay were transformed to control percentages. The results of EC20, EC50 and EC80 values (concentration of test agent that caused a 20% 50% and 80% inhibition in NR, KBP and ATP respectively) were expressed as μM. The ECx values and 95% confidence limits were calculated by computerized probit analysis using the SPSS© 11.5 package.

Experiments were performed in triplicate and six replicates were done for each concentration. The individual data points of the concentration–response cytotoxicity charts are presented as the arithmetic mean percent inhibition relative to the control+/- standard deviation (S.D.). Coefficient of variation (C.V.) for the controls of each test was calculated to ascertain reproducibility. Statistical analyses were carried out using analysis of variance (ANOVA $p<0.05$).

3.2.4. Staining of Acetylcholinesterase (AChE) activity

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AChE activity of RTG-2 and bovine granulosa cells harvested from long-term cell culture was stained according to the method of Karnovsky and Roots (1964).

The harvested cells were fixed for 10 min in 1.5 % paraformaldehyde in PBS, rinsed one time with PBS, and incubated in staining solution [2 mM acetylthiocholine iodide (AcSCh), 5.7 mM sodium citrate, 3.4 mM cupric sulfate, 0.5 mM postassium ferricyanide in 0.1 mM sodium phosphate buffer (pH 6.0)] for 12 h at 20 ºC.

Three treatments were done: Staining solution, Staining solution+ AcSCh, Staining solution+ AcSCh +eserine hemisulfate (selective inhibitor of the activity of all the ChE present in the tissue) in order to observe possible degradation of the substrate AcSCh by other sources different to esterases.

3.2.5. Acetylcholinesterase assay

3.2.5.1. Characterisation of the enzyme

Enzymatic characterization was performed by determining substrate preferences and selective inhibitory effects. The substrate preferences of RTG-2 cell line and bovine granulosa cells were studied by measuring the activity of ChE at increasing concentrations of the substrates AcSCh (acetylthiocholine iodide), BuSCh (s-butyrylthiocholine iodide) and PrSCh (propionylthiocholine iodide). Eserine hemisulfate was used as selective inhibitor of the activity of all the ChE present in the tissue, iso-OMPA as selective inhibitor of pseudocholinesterase (PChE) and BW284C51 as selective inhibitor of AChE. The enzymatic activity was determined with AcSCh after an incubation period of 45 min at 26±1°C. For each chemical, 0.008 ml of a stock solution containing the test-substance was added to 0.492 ml of suspension of homogenated cells.

Controls were incubated with 0.008 ml of ultra-pure water. Additional controls with ethanol were also included when appropriate.

ChE enzymatic activity was determined at 405 nm by the method of Ellman et al. (1961) adapted to microplate as explained down.

3.2.5.2. Cell culture and treatment

The inhibition of the enzyme acetylcholinesterese was measured in RTG-2 cell line and bovine granulosa cells.

Both cells were cultured in MEM supplemented with 10% heat-inactivated foetal calf serum (FCS) and the other medium complements (see annex 1 of material and methods, Table 1).

Cells were seeded at a density of 2 x 10^6 cells/mL in 35 mm Petri dishes. For the experiment, a medium + 1% FCS containig different concentration of the pesticides was added. The final dose of ethanol in medium did not exceed 0.5%.

The cells were incubated at 20ºC or 37ºC for 48 h in presence of the pesticide.

3.2.5.3. Acetylcholinesterase determination

After incubation with the pesticides, cells were scrapped from the petri dishes, homogenized in cold 100 mM phosphate buffer with pH 7.4 during 1 min using a stainless steel Polytron homogenizer and kept in ice until analysis.

Enzyme activity was immediately determined in duplicate according to the colorimetric method of Ellman et al. (1961) at 25 ºC but adapted to a 96-well microplate as described in Guilherminho et al., 1996 and Barata et al., 2001.

Acetylthiocholine iodide (AcSCh) was used as sustrate with 5,5-dithiobis-2-nitrobenzoate (DTNB) as the thiol indicator. The reaction mixture contained: 30 ml phosphate buffer (pH 7.4, 100 mM), 1.2 ml DTNB (6.24 mM in 100 mM phosphate buffer, pH 7.4) and 1.5 ml AcSCh (10.27 mM in ultrapure water). The reaction was initiated by addition of 200 μl of the reaction mixture and an aliquot of 100μl of cellular suspension in each well.

Final concentrations in the reaction mixture were: AcSCh, 0.34 mM; DTNB, 0.17mM. All the chemicals were obtained from Sigma.

The content in every well was mixed and the absorbance read at 5, 10 and 15 min in a spectrophotometer Bio-tek E312 microplate reader at 405 nm.

3.2.5.4. Protein determination

Results were referred to the protein content of the cells, which was determined in quadriplicate by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.

Cells specific cholinesterase activity was reported as nmol AcSCh hydrolyzed per milligram protein per minute and protein content as milligram per ml.
The percentage of AChE inhibition was derived by expressing the activity levels of exposed cells as a percentage of the activity in cell control.

3.2.5.5. Statistics

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Experiments were carried out in triplicate in different days and each concentration was done in duplicate for AChE determinations and in quadriplicate for protein determination. Values of 50% acetylcholinesterase inhibition concentration (IC50) were calculated using the SPSS© 11.5 package. Results are presented as means ± standard desviation (S.D.). Statistical analyses were performed using EXCEL software to determine the one way analysis of variance and assess statistical significance (ANOVA). Differences between means were considered significant for $P<0.05$. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot).

3.2.6. Genotoxicity. Comet assay

Genotoxicity was measured by means of the single-cell gel electrophoresis or comet assay. This assay is a promising genotoxic indicator method since it allows the rapid, relatively simple, and sensitive measurement of DNA damage in eukaryotic cells (McKelvey et al., 1993; Fairbairn et al., 1995; Tice et al., 2000).

3.2.6.1. Exposure of cells to pesticides

Confluent cultures were trypsinized, centrifuged, and cells were counted in a hemocytometer to give a cell suspension of 1.25x10^5 cells/mL. From this suspension, a volume of 200 μL was given into each well of a 96-well plate Costar.

After a 24 h period, the culture medium was removed from the wells, and the attached cells were washed twice with 200 μL per well of phosphate-buffered saline PBS containing Ca2+ and Mg2+. Then, 200 μL of serum-free MEM culture medium containing the various dilutions of the test compounds in constant volumes of vehicle solvent were added and the cells were incubated for 48 h. Negative controls contained no toxicant, but only solvent (ethanol).

To identify the non-cytotoxic concentration range suitable for genotoxicity testing, we evaluated, before performing the genotoxicity assays, the cytotoxicity of the test compounds by means of the neutral red uptake inhibition assay NR, inhibition of the cellular detachment Kenacid blue protein assay and ATP determination as described above.

3.2.6.2. Comet assay

After the 48 h exposure period, the medium was removed from the wells, the microplates were placed on a tray over ice, and cell layers were washed twice with 4 mL of cold 1x PBS w/o Ca2+ and Mg2+, and then trypsinized with 90 μL per well of a 1-mg/mL trypsin (Sigma) solution to obtain a suspension of single cells.

Trypsinization was stopped with 90 μL per well of medium with FCS; Cells were transferred to centrifuge tube and after a centrifugation of 5 min at 2500 rpm the pellets

were washed once in ice cold PBS w/o Ca2+ and Mg2+, and cells were resuspended at 1 x 10^5 cells/ml in ice cold PBS w/o Ca2+ and Mg2+.

Then, 50 μL of the cell suspension were mixed with 500 μL of low melting point agarose LMAgarose, Trevigen, Germany at 35ºC, and 75 μL of this agarose suspension were spread by means of a coverslip on a CometSlide™ specially treated to promote adherence of low melting point agarose. This eliminates the time consuming and unreliable traditional method of preparing base layers of agarose.

The slides were placed flat at 4°C in the dark for 10 minutes. Then, the slides were immersed in prechilled Lysis Solution (Trevigen, Germany) and left on ice, for 30 minutes to 60 minutes.

After this period, slides were immersed in freshly prepared Alkaline Solution, pH>13 (300 mM NaOH, 1mM EDTA) for 20 to 60 minutes at room temperature, in the dark.

Slides were transferred to a horizontal electrophoresis apparatus and were placed flat onto a gel tray and align equidistant from the electrodes. Electrophoresis was then carried out at 25V (electric potential = 0.833 V/cm) and at starting current of approximately 300 mA for 20-40 minutes. After electrophoresis, the slides wee placed on a staining tray, were neutralized with 0.4 mol/L TRIS, pH 7.5 and immersed in 70% ethanol for 5 minutes. Then, they were dried with air. Drying brings all the cells in a single plane to facilitate observation.

The slides were stained with 50 μL of diluted SYBR® Green I (Trevigen, Germany) and viewed under an epifluorescence microscopy (Olympus BX51) at 40x magnification equipped with an excitation filter of 494-515nm and a barrier filter of 590 nm.

3.2.6.3. Qualitative analysis

The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for healthy cells. Scoring can then be made according to nominal, medium or high intensity tail DNA content. At least 75 cells were scored per sample.

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3.2.7. Aromatase activity

3.2.7.1. Progesterone (P4) determination

Progesterone production (i.e., functional aromatase activity) was assessed adding to the treatment media a standard quantity of testosterone (1 µg/mL) used as a progesterone precursor.

The assay was performed in Italy using miniVIDAS, BioMerieux instrument in collaboration with Dr. Francesca Caloni and Dr. Fausto Cremonesi of the University of Veterinary of Milano, Department of Science and Technology for the Food Safety (VSA) and the Department of Clinics

Approximately 3 x10^5 cells/ml viable granulosa cells and 1x10^5 cells/ml RTG-2 were added to 96-microwell plates in a total volume of 200 µL medium. After 48 h of culture, medium was changed for medium supplemented with carbofuran, chlorpyrifos or the mixture of them in different final concentrations. Controls were incubated with vehicle treated medium alone.

*After the incubation period (48 h), medium was collected (200 µL) and stored at -80 ºC for progesterone (P4) determination. Results were reported in nanograms/ml/n.cell for granulosa cells and nanograms/ml/n.cell*100 for RTG-2 cells.*

3.2.7.2. Statistics

Each experiment was performed three times with different pools of granulosa cells collected from 4 to 8 ovaries of each pool and with different pools of RTG-2 cells.
Each well was a replicate and each experiment contained at least two replicates per treatment.

Experimental data are presented as the arithmetic mean +/- standard deviation (S.D.) of measurements from duplicate culture wells from three experiments. Coefficient of variation (C.V.) for the controls of each test was calculated to ascertain reproducibility.

3.3. In vivo assays

3.3.1. Fish. *Danio rerio*

Juvenile zebra fish, Danio rerio (Figure 10) of weight 0.4 ±0.1 g and size 2.5±0.5 cm, of both sexes were purchased from commercial fish farms and maintained in 100 L tanks containing oxygenated nonchlorinated freshwater. Experiments began after an acclimatization period of at least 2 weeks.

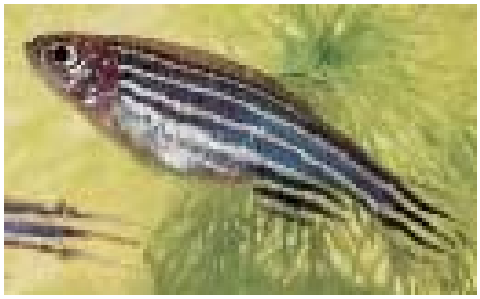

Figure 10. Danio rerio

During holding, fish were maintained in the laboratory with running nonchlorinated tap water under constant environmental conditions. Water quality characteristics were: pH at 7.4-7.6, hardness between 300-400 mg/L and constant aeration at 8.5 mg/l of oxygen. A light: darkness photoperiod 16:8 was applied. Temperature was kept at 20 ± 2 ºC. Commercial feed (Tetramin) was given three times a week.

3.3.1.1. Acute toxicity assay. Mortality

For all experiments, the static test was performed as described by the European Organization for Cooperation and Development (OCDE, 1993), modified for measuring brain, muscle and liver AChE activity.

Briefly, the stocking rate for the in vivo study was 5 fish/aquarium (each aquarium had 40cm length, 35cm width and 20cm depth). Two aquaria were used per treatment (OECD, 1993). Fish were not fed for 24h prior to the experiments and no food was provided during the test period.

Fish bioassays were made without removal of either pesticides or the aquatic medium, for 96 h using a static bioassay system.
Carbofuran and chlorpyrifos were dissolved in absolute ethanol in order to prepare stock solutions. These solutions were further diluted in ASTM hard water (ASTM, 1980) to obtain the experimental concentrations in aquariums.
Acute toxicity was evaluated by exposing fish to several concentrations of single pesticides and of a mixture of chlorpyrifos/carbofuran.

In concrete, four trial dosages were selected according to the toxicity of the pesticides on other fishes described in the literature, (Dembélé et al., 2000; Rendón-von Osten et al., 2005; Bretaud et al., 2000) and a preliminary and definitive experiment was conducted on zebra fish. Groups of seven individuals were exposed to those concentrations for 96 h in separate glass aquariums. LD50 values were determined after the exposure.

Control animals were maintained in a pesticide-free medium containing 0.25 ml/L of ethanol, because the pesticides were dissolved in ethanol.
An ASTM hard water control was also included in the experimental design.

In the acute toxicity tests, mortality was considered as the endpoint. Mortality was defined as absence of breathing movements (gill and mouth) in fish, and absence of cardiac beat and blood circulation determined by stereoscopic microscopy.

Fish were observed daily for mortality and clinical signs of toxicity.
As result of the AChE depression, fish displayed motor hyperactivity and erratic jumping at the onset of treatment.

At the end of the bioassay (96h) effects on the biomarker AChE were analyzed, the surviving fish were decapitated and the brain, muscle and liver were removed and kept individually at 0ºC (immersed in an ice bath) for determination of AChE activity and protein quantity. Bocquené et al., 1990 have demonstrated that only minor losses in AChE activity occur under these conditions.

3.3.1.2 Acetylcholinesterase assay

3.3.1.2.1. Tissue preparation

Fish were placed on a cold glass plate. After decapitation with a scissors, the whole brain was excised. Dorsal muscle and liver are removed too (care was taken to avoid any blood clots, bones, skin or portions of nerves), and were immediately deep-frozen in liquid nitrogen and then stored at -80°C in 1.5-ml polypropylene Eppendorf microcentrifuge tubes until the further analysis. Tissue AChE activity measurements were performed individually.

Prior to the analysis, the brain, muscle and liver samples were weighed and after thawing were homogenized in cold 100 mM phosphate buffer with pH 7.4 (20 mg tissue per ml of buffer) using a stainless steel Polytron homogenizer.

Homogenates were centrifuged at 4ºC for 10 min at 10000 g in a refrigerated centrifuge (Hettich Mikro 22R) and the supernatants were transferred to clean test tubes and used for cholinesterase and protein assays.

Before enzyme determination, the supernatant was diluted with 100 mM phosphate buffer (pH 7.5), depending on fish tissue enzyme activity: brain 10-fold, muscle 5-fold, liver directly.

3.3.1.2.2 Characterisation of the enzyme

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Enzymatic characterization was performed by determining substrate preferences and selective inhibitor effects. The substrate preferences of D. rerio were studied by measuring the activity of ChE at increasing concentrations of the substrates AcSCh (acetylthiocholine iodide), BuSCh (s-butyrylthiocholine iodide) and PrSCh (propionylthiocholine iodide). Eserine hemisulfate was used as selective inhibitor of the activity of all the ChE present in the tissue, iso-OMPA as selective inhibitor of pseudocholinesterase (PChE) and BW284C51 as selective inhibitor of AChE. The enzymatic activity was determined with AcSCh after an incubation period of 45 min at

26 ± 1 °C. For each chemical, 0.008 ml of a stock solution containing the test-substance was added to 0.492 ml of D. rerio homogenate.
Controls were incubated with 0.008 ml of ultra-pure water. Additional controls with ethanol were also included when appropriate.

ChE enzymatic activity was determined at 405 nm by the method of Ellman et al. (1961) adapted to microplate as described in Guilherminho et al., 1996 and Barata et al., 2001.

3.3.1.2.3. Acetylcholinesterase determination

Enzyme activity was immediately determined in duplicate according to the colorimetric method of Ellman et al. (1961) at 25 ºC but adapted to a 96-well microplate. Acetylthiocholine iodide (ATCh) was used as sustrate with 5,5-dithiobis-2-nitrobenzoate (DTNB) as the thiol indicator. The reaction mixture contained: 30 ml phosphate buffer (pH 7.4, 100 mM), 1.2 ml DTNB (6.24 mM in 100 mM phosphate buffer, pH 7.4) and 1.5 ml ATCh (10.27 mM in ultrapure water). The reaction was initiated by addition of 200 μl of the reaction mixture and an aliquot of 100 l of supernatants in each well.
Final concentrations in the reaction mixture were: ATCh, 0.34 mM; DTNB, 0.17mM. All the chemicals were obtained from Sigma.

The content in every well was mixed and the absorbance read at 5, 10 and 15 min in a spectrophotometer Bio-tek E312 microplate reader at 405 nm. The rate of TNB production evaluated during 5, 10 and 15 min at 405 nm was used to estimate spontaneus substrate hydrolysis.
Results were refered to the protein content of the supernatants, which was determined in quadriplicate by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard.
Brain, muscle and liver specific cholinesterase activity was reported as nmol ATCh hydrolyzed per milligram protein per minute and protein content as milligram per ml. The percentage of AChE inhibition was derived by expressing the activity levels of exposed animals as a percentage of the activity in controls.

3.3.1.2.4. Statistics

Experiments were carried out in duplicate in different days and each concentration was done in duplicate for AChE determinations and in quadriplicate for protein determination.
Values of 50% lethal concentration (LC50), 50% acetylcholinesterase inhibition concentration (IC50) were calculated using the SPSS© 11.5 package.

Results are presented as means ± standard desviation (S.D.). Statistical analyses were performed using EXCEL software to determine the one way analysis of variance and assess statistical significance (ANOVA). Differences between means were considered significant for P<0.05. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot).

3.3.2. Microalgae

3.3.2.1. Growth inhibition of microalgae

The acute toxicity tests with algae were carried out with the freshwater unicellular green algae S. subspicatus (CCAP 276/20), S.capricornutum (CCAP 278/4) and C. vulgaris (CCAP 221/11B) obtained from the Culture Collection of Algae and Protozoa, England.

Figure 11. Chlorella vulgaris. Contrast phase 100x

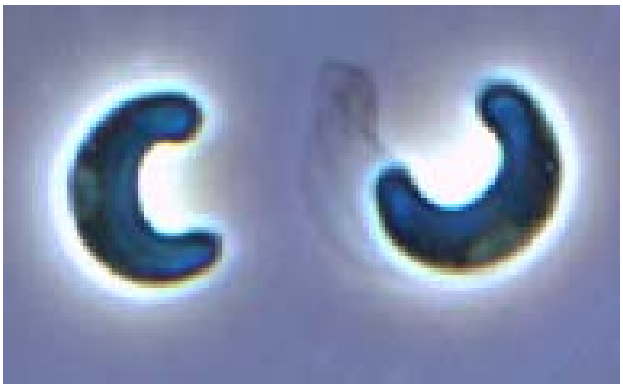


Figure 12. *Selenastrum capricornutm*. Contrast phase 100x

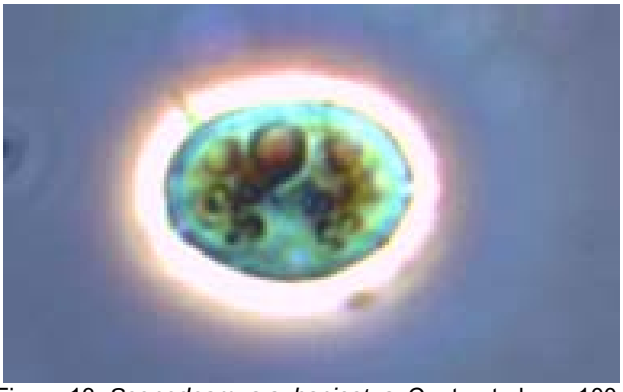


Figure 13. *Scenedesmus subspicatus*. Contrast phase 100x

3.3.2.2. Nutrient medium

The medium for the algal growth inhibition test with S. subspicatus, S. capricornutum and C. vulgaris was prepared in accordance with OECD Test Guideline 201, using a medium composed of distilled water and the chemical ingredients described in annex 4 of the material and methods in Table 2.

3.3.2.3. Test methods

The single-celled algae were propagated photoautotrophically in a 250 mL Erlenmeyer flask containing 100 mL liquid and kept on a rotator shaker (100 rpm) at 25°C, illuminated with cool white fluorescent lights at a continuous light intensity of 5000 lx.

An aliquot of algae was inoculated once a week into a new culture medium with salts. Every 8-10 week the cultures were renewed from algal cells cultured in 4 % agar solid medium.

All procedures, the inoculation of new media and the culture handling were made under sterile conditions.
Culture medium was prepared for the bioassay using an aliquot of the stock culture medium of 6 days old in their growth exponential time.

In the algal growth-inhibition experiments, 10 mL aliquots of the algae medium containing 1mL of single algal cells corresponding to an initial cell density of 5 x 10^4 cell/ml of each specie were distributed to sterile tubes. The media of S. subspicatus, S.capricornutum and C. vulgaris were then treated with various pesticide concentrations and incubated for 72 h on an orbital shaker (100 rpm) at a temperature of 25°C with a continuous light intensity of 5000 lx (Wijk et al., 1998; Oliveira-Filho and Paumgartten, 2000; Ma, 2002). A wide range of concentrations in a previous test was assessed in order to find the adequate range of toxicity for each pesticide. Then, similar doses were used according to the previous test (Moreno-Garrido et al., 2000). Growth of algal cells was calculated indirectly using optical density at data in this work.

Cell counts were correlated with absorbance over time for 72 h on a Dr. Lange spectrophotometer. The most suitable wavelength to use for monitoring culture growth

was 666 nm. There was a highly significant relationship between cell numbers and OD 666 nm for C. vulgaris and S. subspicatus and S. capricornutum according to studies of Ma et al (2002a); Ma et al. (2003) and Ma et al. (2004)). Thus, growth of algal cells was calculated indirectly using optical density data in this work.

Appropriate control systems containing no pesticide were included in each experiment. Control and treated cultures were grown under the same conditions of temperature, photoperiod, and shaking of the stock cultures and were done in triplicate. Each toxicity test was done twice. In each experiment, percentage inhibition values, relative to growth in control systems, were calculated using optical density data.

3.3.2.4. Statistics

Absolute values of each assay were transformed to control percentages. Each experiment was done in duplicate, and triplicates were done for each concentration. The EC50 values (pesticide concentration required to cause a 50% reduction in growth) and 95% confidence limits were calculated using linear regression analysis of transformed pesticide concentration as natural logarithm data versus percentage inhibition with logit method using the SPSS© 11.5 package.

The EC20 and EC80 values are the concentrations giving rise to 20% and 80% reduction in growth, respectively, compared with the control and they were calculated using linear regression analysis in the same ways as for computing EC50 values.

3.3.3. Bacteria. *Vibrio fischeri*

3.3.3.1. Bioluminiscence inhibition

The Microtox® test was performed using the Microtox Model 500 Toxicity Analyzer. The freeze-dried bacteria (approx. 10^6 cells), reconstitution solution (organic-free distilled water), diluent (2% NaCl) and adjustment solution (non-toxic 22% sodium chloride dilution) to provide osmotic stability for the marine bacterium, were obtained from Microbics corporation (Carlsbad, CA, USA).

The Microtox test is based on the measurement of light production inhibition by organisms. The basic procedure recommended in the instrument manual was used for the toxicity test (Microbics 1989). All samples were adjusted to 2 ± 0.2% (v/v) NaCl and the tests were run at 15°C. The incubator block and the chamber in which light production is measured were maintained at this temperature of 15ºC +/- 0.1ºC. Light measurements were taken at 5 and 15 min.

The Microtox® experimental protocol and materials were evaluated with a standard phenol test experiment and the EC50 was determined to be 27% for 5 min and 28% for 15 min of analysis, which is well within the manufacturers recommended range.

The concentration of the organic solvent did not exceed 8% (v/v) in the test samples. Our preliminary studies showed that concentrations of solvent vehicles exceeding 8% were toxic to the bacteria.

3.3.3.2. Stadistics

"

Absolute values of each assay were transformed to control percentages. Each test was carried out by triplicate. The EC50 values (expressed in μM) differed by not more than 20% from each other.

Graphic plots of the Γ values (defined as the quotient between the lost light and the light remaining) versus pesticide concentration were made. Standard statistics are used to calculate confidence intervals for this estimate (see details in annex 5 of the material and methods).

Results

4. Results

In vitro results

4.1. RTG-2 cell line (*Oncorhynchus mykiss*)

4.1.1. Cytotoxicity

Firstly, we examined changes in cell morphology of the RTG-2 cell line derived from rainbow trout (Oncorhynchus mykiss) after an exposure time of 48h to chlorpyrifos, carbofuran and the mixture are represented in Figures 14, 15, 16, respectively. We can observe that in the three exposures, the control cells (unexposed to pesticides and maintained only in MEM) display always a fully confluent monolayer (Figure 14, 15, 16 a). Furthermore, cells exposed to 1 μM of chlorpyrifos, 0.45 μM of carbofuran and 0.45 μM of their mixture show a discernible change in the density of the monolayer. There is a little difference respect to control. We only observe a subtle change in the cell density of the monolayer compared to control cells (Figure 14, 15, 16 b). When the concentration of pesticide increases, the morphological changes are bigger compared to control cells. At 10 μM of chlorpyrifos, carbofuran and mixture cells display breaks in the cell monolayer (Figure 14, 15, 16 c). In contrast, cells exposed to 100 μM of chlorpyrifos, carbofuran and their mixture (Figure 14, 15, 16 d) show evident changes in cell shape. At that concentration the maximum effect is observed and cell damage is obvious because the integrity of the cell monolayer is lost.

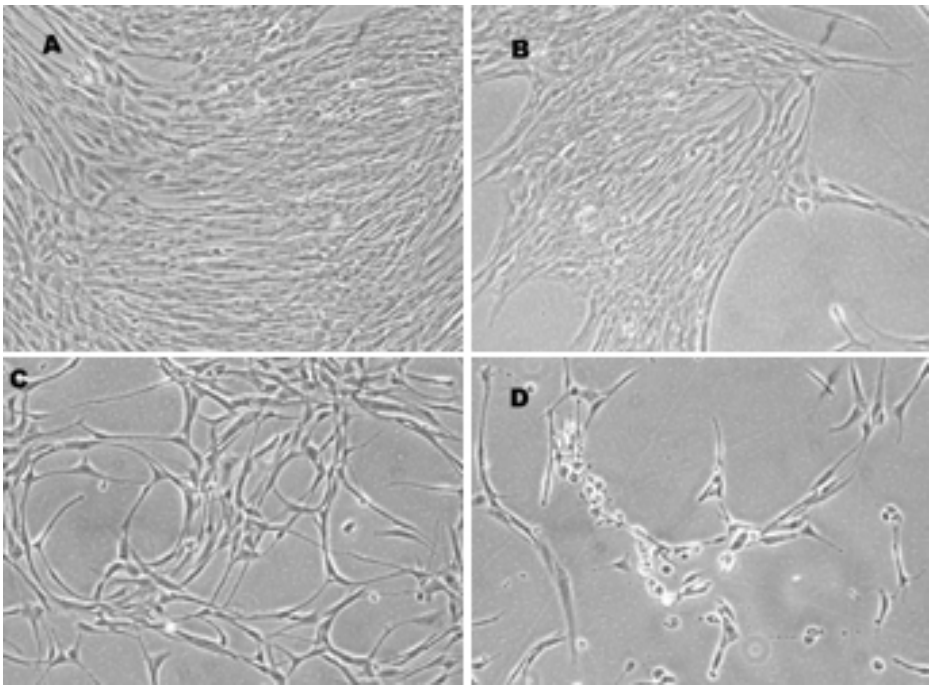


Figure 14. Light microscopy appearance of RTG-2 cell line following exposure to chlorpyrifos: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 1μM of chlorpyrifos showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of chlorpyrifos illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

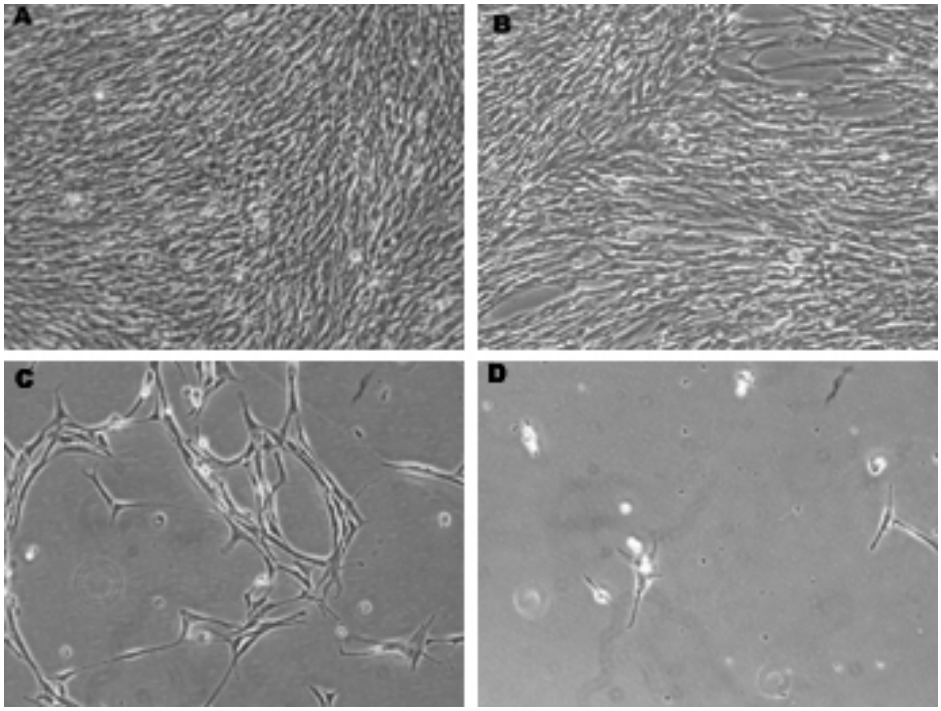


Figure 15. Light microscopy appearance of RTG-2 cell line following exposure to carbofuran: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 0.45 μM of carbofuran showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of cabofuran illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

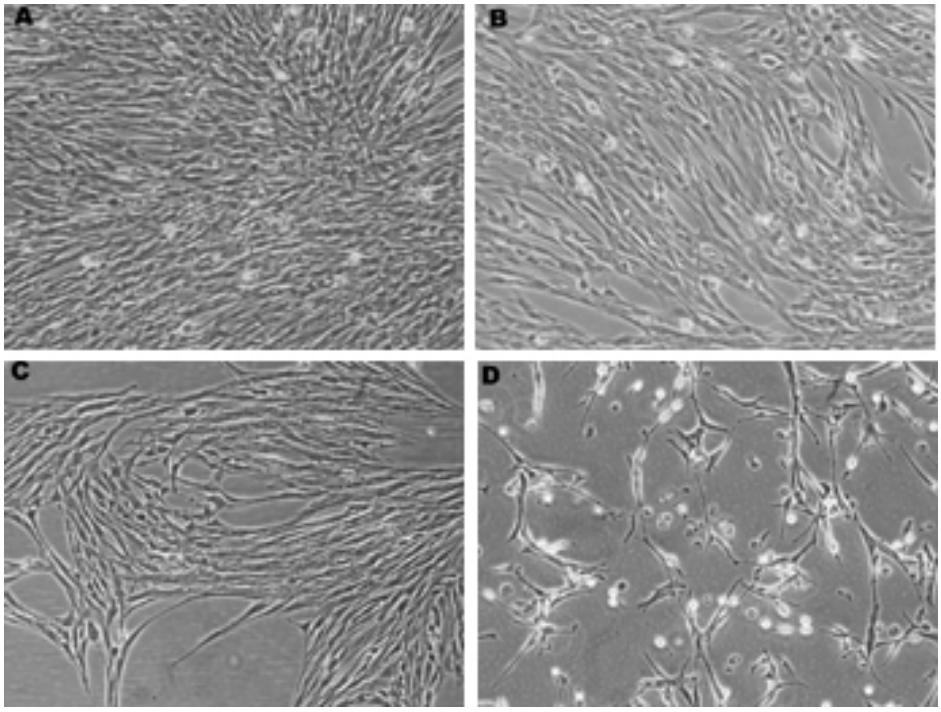


Figure 16. Light microscopy appearance of RTG-2 cell line following exposure to mixture chlorpyrifos-carbofuran: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 0.45 μM of the mixture showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of mixture illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

After checking the appearance of RTG-2 cells by a light microscopy the respective HTD values for chlorpyrifos, carbofuran and the mixture were reported and are shown in Table 1 expressed in μM. The HTD is defined as the highest test pesticide concentration that causes the minimum morphological changes to cells (Shúilleabháin et al, 2004; Liebsch and Spielmann, 1995). As we could appreciate in the microscopic observation (Figure 14, 15, 16 b) the minimum effects start at 1 μM of chlorpyrifos, 0.45 μM of carbofuran and 0.45 μM of their mixture. Therefore, carbofuran and the mixture are more toxic than chlorpyrifos because a lower concentration (0.45 μM) is needed to cause the same subtle changes than 1 μM of chlorpyrifos.

Table 1. Values of pesticide that causes the minimum morphological changes to RTG-2 cell line after an exposure to 48h to chlorpyrifos, carbofuran and a mixture chlorpyrifos-carbofuran expressed in μM.

Pesticide	**HTD (μM)**
Chlorpyrifos	*1*
Carbofuran	*0.45*
Mixture	*0.45*

Secondly, the cytotoxicity of each pesticide was measured using three enpoints, the Neutral Red assay (NR), the Kenacid Blue Protein assay (KB) and the Adenosin triphospate determination (ATP).

Cell viability was measured after 3 h of incubation to the dye neutral red, the lysosoms of the living cells incorporated the dye and presented the appearance showed in Figure 17.

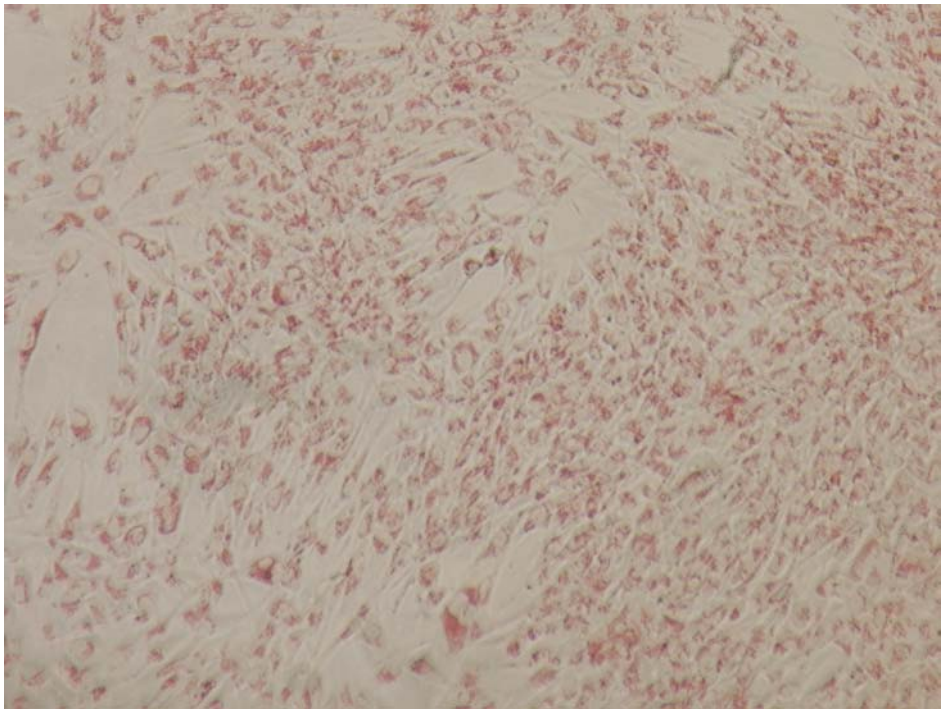


Figure 17. Light microscopic appearance of RTG-2 cell line following 3 h of incubation to neutral red dye. The living cells incorporate the colorant into the lysosoms. Magnification: 10 x

The inhibition of cell proliferation was measured by the changes in total cell protein by the Kenacid Blue dye binding method. After 30 min of incubation to the dye Kenacid Blue the cells were stained as shows Figure 18.

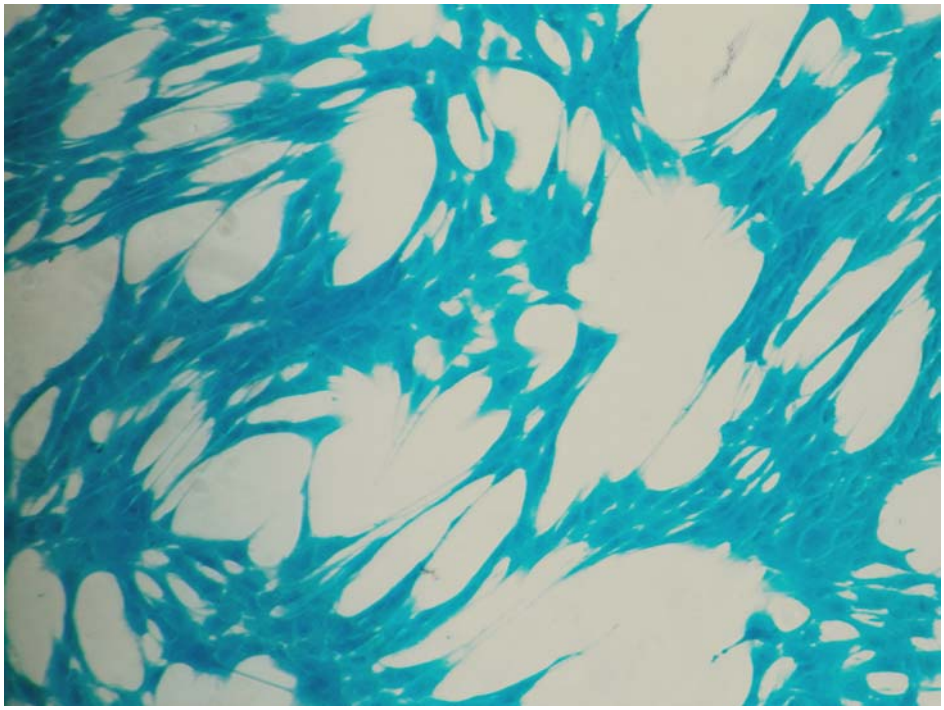


Figure 18. Light microscopic appearance of RTG-2 cell line after 30 min of incubation to Kenacid Blue dye. Magnification: 40 x

The obtained cytotoxicity values using the three endpoints were compared for several concentrations of chlorpyrifos, carbofuran and their mixture and were represented in Figure 19 a-c. Values are expressed as percentage to control cells.
We observe a correlation between these endpoints. In general, there is a tendency being the neutral red uptake and the ATP content the most sensitive endpoints and protein determination the less sensitive, but there are no statistical differences.

a)

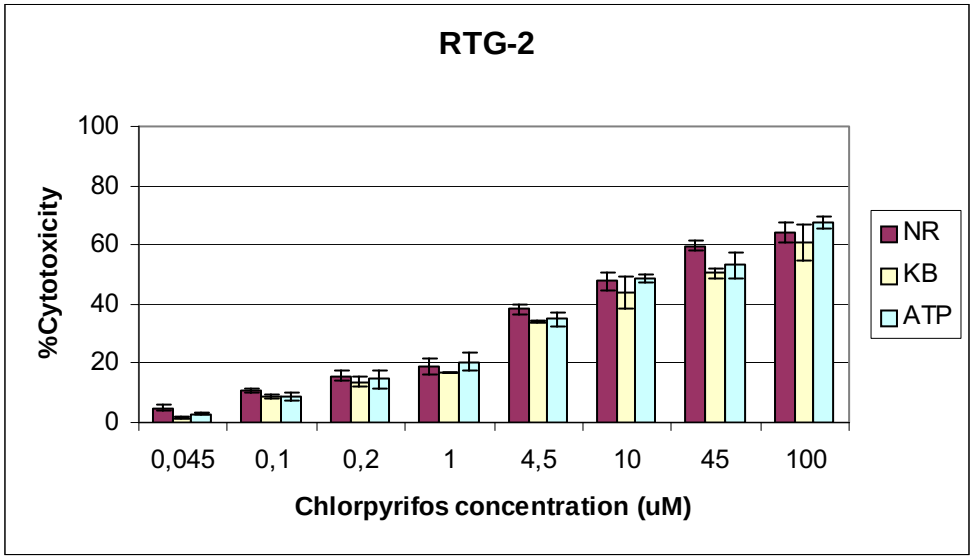


Figure 19-a. Cytotoxicity of chlorpyrifos to RTG-2 cells as determined by 48h NR, KB, ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments

b)

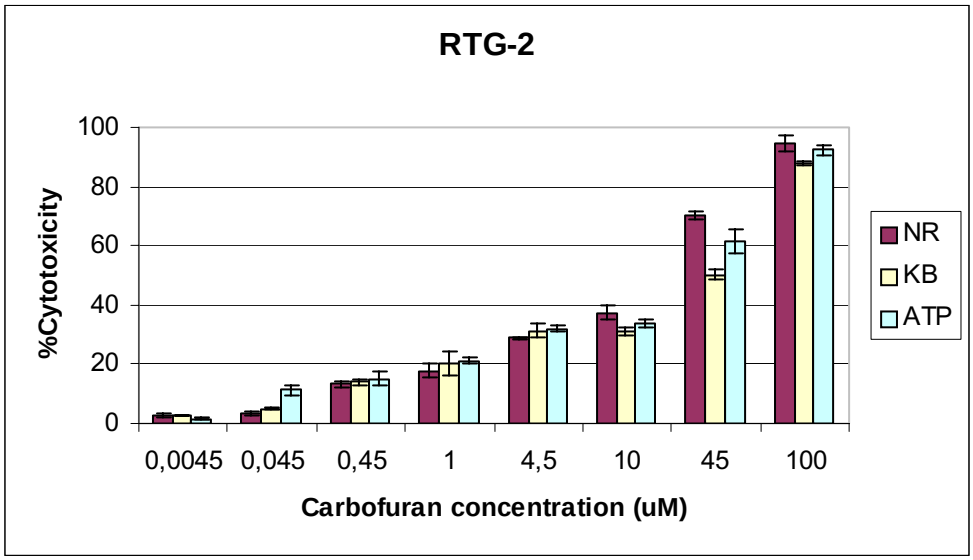


Figure 19-b. Cytotoxicity of carbofuran to RTG-2 cells as determined by 48h NR, KB, ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments.

c)

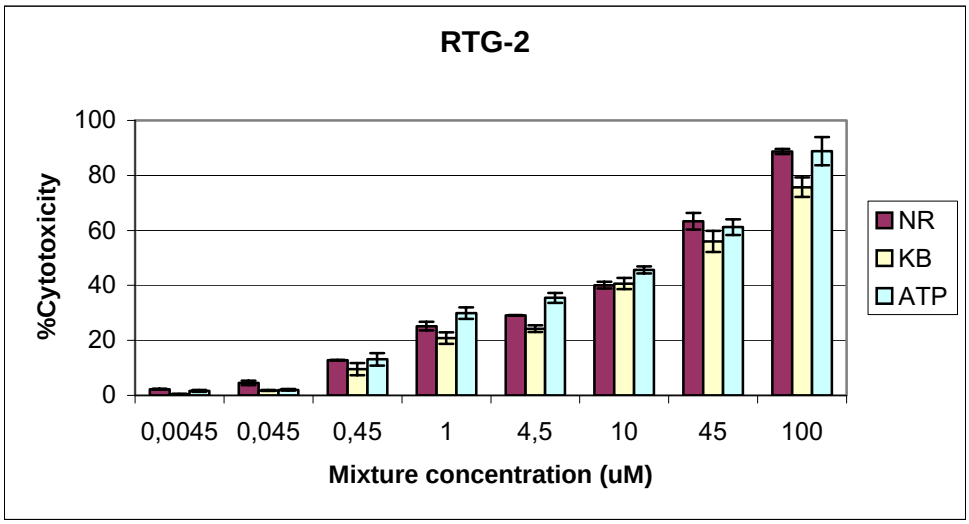


Figure 19-c. Cytotoxicity of mixture chlorpyrifos-carbofuran to RTG-2 cells as determined by 48h NR, KB and ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments. Mixture values are the sum of the concentration of chlorpyrifos and carbofuran in test solutions (concentration chlorpyrifos=concentration carbofuran).

The percentages of cytotoxicity in RTG-2 fish cell line related to the used pesticide concentrations after 48 h of exposure are shown in Table 1 of the annex 1 of results. Table 2 shows the obtained EC20, EC50, EC80 values and its 95% confidence intervals expressed in μM for the tested pesticides using the three endpoints, Neutral red uptake (NR), Kenacid blue protein (KB) and ATP measurement. In order to integrate the three endpoints in a single data, a cytotoxicity index (CI) was determined by the equation of Castaño et al., 1994b, and it is also reported in Table 2. According to values presented in Table 2, carbofuran seems more toxic than chlorpyrifos (there are not statistical differences except for the EC80%). The concentration that causes 20% of cytotoxicity according to the cytotoxicity index (CI) is 1.08 μM for chlorpyrifos, 1.03 μM for carbofuran and 1.38 μM for the mixture. The concentration that causes the 50% of cytotoxicity is 35.95 μM for chlorpyrifos, 19.36 μM for carbofuran and 21.23 μM for the mixture. The concentration that causes the 80% of cytotoxicity is 1198 μM for chlorpyrifos, 365 μM for carbofuran and 325 μM for the mixture.

Table 2.. Obtained values of a) EC20, b)EC50, c)EC80 and its 95% confidence intervals expressed in μM for the tested pesticides using the in vitro endpoints and the Castaño cytotoxicity index. NR=Neutral Red, KB= Kenacid Blue, ATP= Adenosin triphospate. CI= Cytotoxicity index. Mixture has the same concentration as single test. Each pesticide was tested in a separate plate to avoid interferences between compounds. Two plates for each pesticide were performed with six replicates for NR and KB and two for ATP.

a)

RTG-2								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC20 (μM)	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**
Chlorpyrifos	*0,91*	*(0,36-1,78)*	*1,65*	*(0,56-3,53)*	*0,60*	*(0,22-1,23)*	*1,08*	*(0,75-1,49)*
Carbofuran	*0,61*	*(0,42-0,84)*	*0.87*	*(0,34-1,72)*	*0,79*	*(0,56-1,07)*	*1,03*	*(0,50-1,78)*
Mixture	*0,20*	*(0,45-1,44)*	*1,63*	*(1,22-2,12)*	*0,80*	*(0,43-1,29)*	*1,38*	*(1,01-1,82)*

b)

RTG-2								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC50 (μM)	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**
Chlorpyrifos	*17,62*	*(12,93-24,95)*	*30,13*	*(21,51-44,43)*	*19,50*	*(14,43-27,36)*	*35,95*	*(25,05-54,83)*
Carbofuran	*9,80*	*(5,36-19,89)*	*17,38*	*(9,16-39,48)*	*10,94*	*(5,75-24,00)*	*19,36*	*(11,37-37,34)*
Mixture	*11,66*	*(7,38-19,71)*	*20,53*	*(16,06-26,90)*	*9,82*	*(6,50-15,52)*	*21,23*	*(16,31-28,45)*

c)

RTG-2								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC80 (μM)	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**
Chlorpyrifos	*105,37*	*(45,26-396,27)*	*1184,19*	*(282,66-16743,51)*	*198,04*	*(73,82-940,29)*	*1198,13*	*(618,88-2727,73)*
Carbofuran	*507,7*	*(290,98-1001,11)*	*344.67*	*(122,39-1777,04)*	*483,19*	*(283,26-924,45)*	*365,51*	*(151,94-1315,28)*
Mixture	*157,49*	*(78,01-419,34)*	*257,89*	*(170,91-423,03)*	*120,43*	*(64,77-278,90)*	*325,55*	*(207,61-560,55)*

4.1.2. Acetylcholinesterase activity staining

Acetylcholinesterase (AChE) activity of RTG-2 cell line was stained according to the method of Karnovsky et al. (1964).

Three treatments were done; results were reported in Table 3.

Treatment 1= Staining solution,

Treatment 2= Staining solution + substrate acetylthiocholine (AcSCh),

Treatment 3= Eserine hemisulfate (an inhibitor of the esterases) was added to the staining solution + AcSCh in order to observe a possible degradation of the substrate AcSCh by other sources different to esterases.

Table 3. Acetylcholinesterase activity staining in RTG-2 cell line.

Acetylcholinesterase activity staining		
Treatment 1	*Staining solution*	*No colour observed*
Treatment 2	*Staining solution+AcSCh*	*Colour observed*
Treatment 3	*Staining solution+ AcSCh +eserine hemisulfate*	*No colour observed*

The appearance of the cells was assessed by light microscopy. Figure 20-a, which corresponds with treatment 1 shows the control cells, there is no colour observed due to the absence of substrate AcSCh.

In contrast, in the treatment 2 (Figure 20-b), there is a formation of a brown precipitate that shows AChE activity.

In treatment 3 (Figure 20-c), with presence of eserine hemisulfate, there is not formation of the brown precipitate indicating that all the colour produced in treatment 2 is due to the activity of esterases.

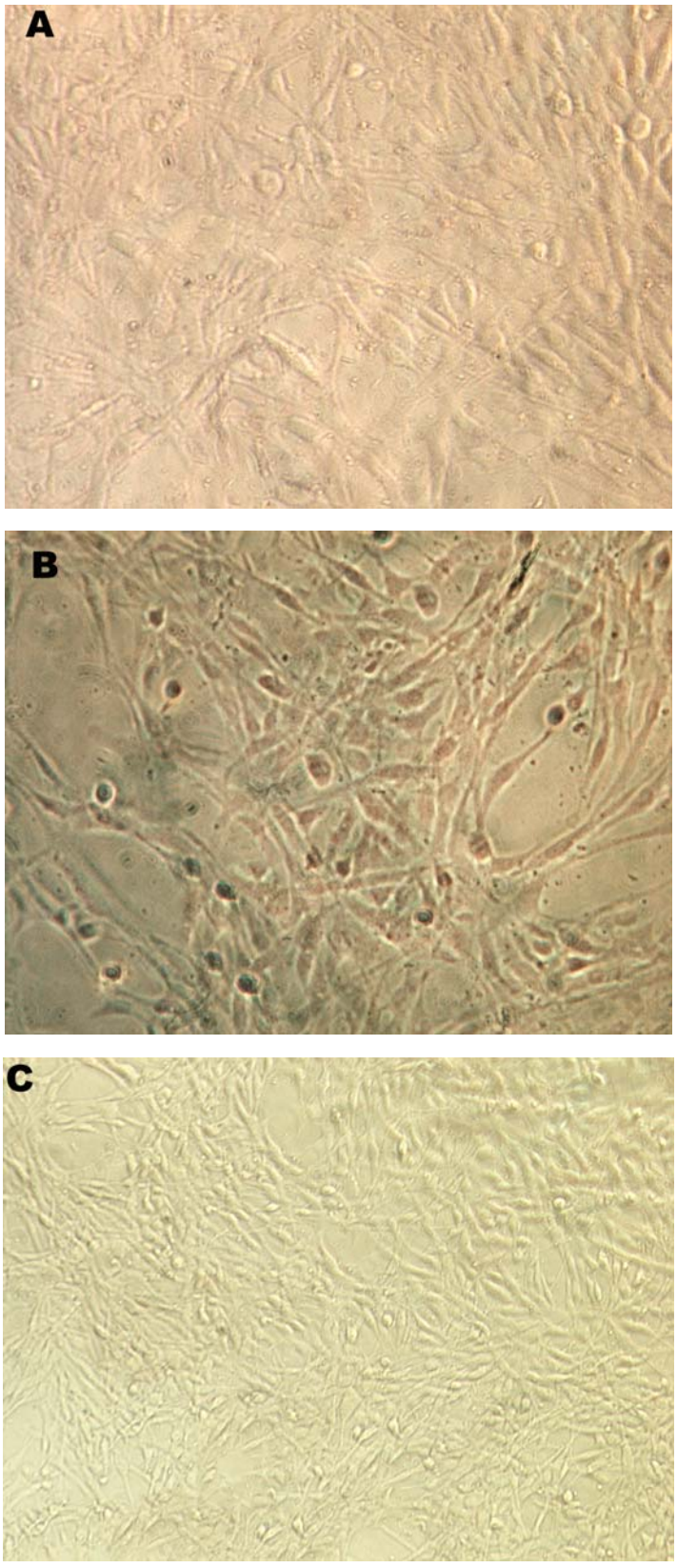


Figure 20. Acetylcholinesterase activity in RTG-2 cells: a) Treatment 1: control cells presented negative staining because there was no substrate AcSCh (acetylthiocholine); b) Treatment 2: cells presented positive staining, there is the

formation of a brown precipitate; c) Treatment 3: cells presented negative staining due to the effect of eserine hemisulfate, an inhibitior of the esterases. Magnification: 40 x

4.1.3. Acetylcholinesterase assay

4.1.3.1. Enzyme characterisation

4.1.3.1.1. Substrate specificity

Three substrates were tested Acetylthiocholine (AcSCh), Propionylthiocholine (PrSCh) and Butyrylthiocholine (BuSCh) in order to determine the relative enzyme activities in RTG-2 cell line.

Enzyme activity expressed in nmol/min/mg protein versus function of increasing concentrations of AcSCh, PrSCh and BuSCh expressed in mM is showed in Figure 21. The highest activity was found with AcSCh (1.8 mM). A very low activity was measured for PrSCh (0.94 mM) and BuSCh (0.87 mM). The specifity of the enzyme for the substrate AcSCh suggests that the enzyme is acetylcholinesterase (AChE).

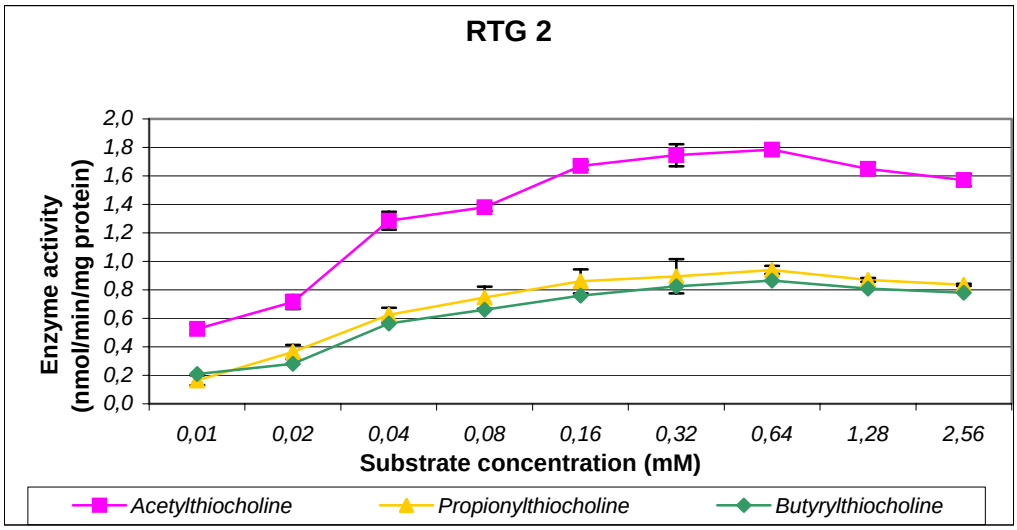


Figure 21 Enzyme activity of RTG-2 cell line expressed as nmol/min/mg prot as function of AcSCh, PrSCh and BuSCh concentration expressed in mM. Values are the mean of three independent experiments with two enzymatic determinations for AChE and three for proteins, corresponding standard error bars were shown.

4.1.3.1.2. Enzyme inhibition

Effects of different inhibitors: eserine hemisulfate, iso-OMPA and BW284C51 on enzyme actitivity of RTG-2 cell line are showed in Figures 22, 23, 24.

Eserine hemisulfate, an inhibitor of all the esterases, significantly inhibited enzyme activity in RTG-2 (Figure 22) after an incubation period of 45 min. Inhibition by eserine hemisulfate is almost complete (88%) at the highest concentration tested (0.1 mM). Similar results were found for BW284C51, a specific inhibitor of acetylcholinesterases, also after an incubation period of 45 min (Figure 24). BW284C51 causes an 80% AChE inhibition at concentration of 0.1 mM. In contrast, RTG-2 cells were relatively insensitive to iso-OMPA, an inhibitor of butyrylcholinesterases, since it only causes a 31.43% inhibition. It suggests that the main enzyme present is acetylcholinesterase.

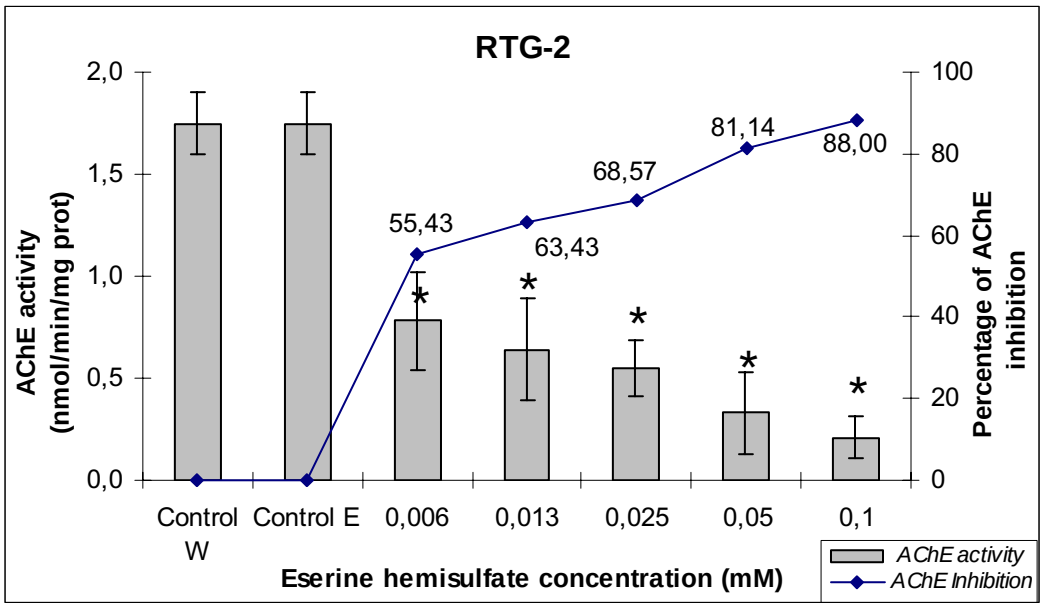


*Figure 22. Effects of eserine hemisulfate (expressed in mM) on enzyme actitivity (expressed in nmol/min/mg prot) of RTG-2 cell line after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

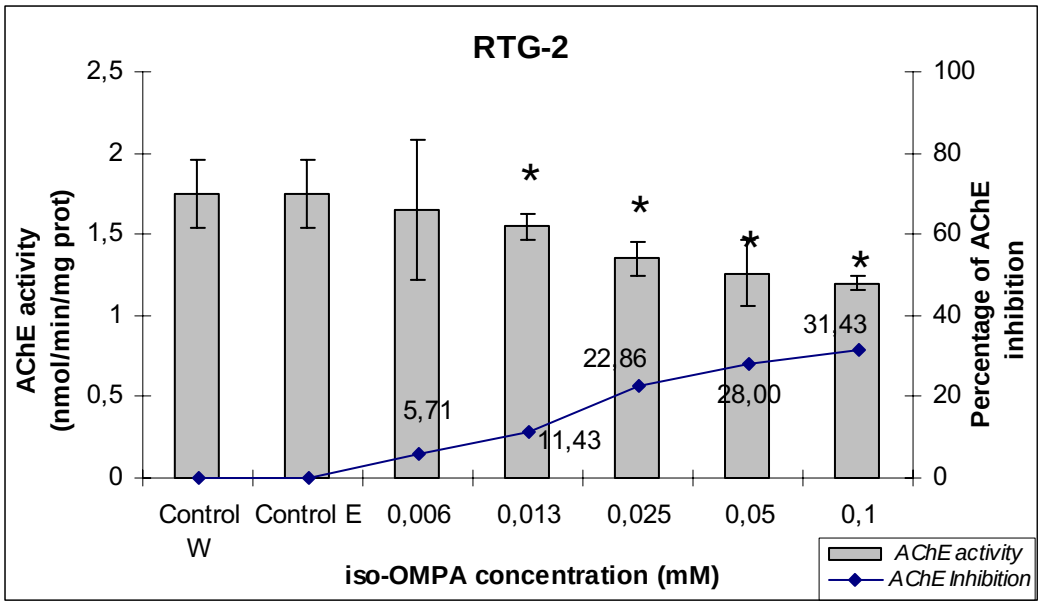


*Figure 23. Effects of iso-OMPA (expressed in mM) on enzyme actitivity (expressed in nmol/min/mg prot) of RTG-2 cell line after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

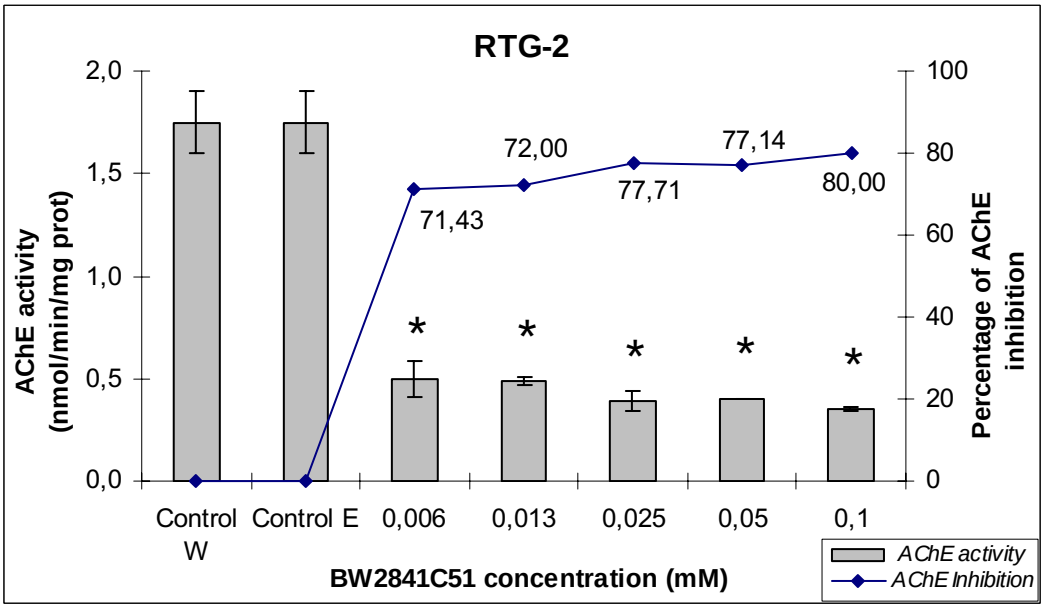


*Figure 24. Effects of BW284C51 (expressed in mM) on enzyme actitivity (expressed in nmol/min/mg prot) of RTG-2 cell line after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

4.1.3.1.3. Optimisation of AChE determination

Effect of substrate concentration on esterase activity expressed in nmol/min/mg protein with acetylthiocholine as substrate in RTG-2 cells and the Michaelis-Menten constant (Km) is shown in Figure 1 of the annex 1 of results.

AChE activity of RTG-2 cell line shows an apparent Michaelian behaviour. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot). The apparent Km value for the substrate AcSCh is 21 μM. The maximum rate of hydrolysis, Vmax, was achieved at 1760 μM (Figure 1 from the annex 1 of results). Inhibition of enzymatic activity was observed at the highest concentration of AcSCh tested, 2.56 mM (Figure 1 from the annex 1 of results).

4.1.3.2. Enzyme determination

The percentages of AChE inhibition in RTG-2 fish cell line related to the used pesticide concentrations after 48 h of exposure are shown in Table 1 of the annex 1 of results. Acetylcholinesterase inhibition values of IC20, IC50, IC80 and its 95% confidence intervals expressed in μM for the tested pesticides are shown in Table 4. Chlorpyrifos inhibits significantly much more acetylcholinesterase activity than carbofuran. The concentration that causes an inhibition of the 20% is 0.14 μM for chlorpyrifos, 1.29 μM for carbofuran and 1.95 μM for the mixture. The concentration that causes an inhibition of 50% of the enzyme activity (IC50) is 1.04 μM for chlorpyrifos, whereas for carbofuran it is 31.44 μM and for the mixture 32.42 μM. The IC80 values are 7.99 μM for chlorpyrifos, 767.57 μM for carbofuran and 538.73 μM for the mixture.

Table 4 Acetylcholinesterase inhibition of RTG-2 a) IC20, b)IC50, c)IC80 and its 95% confidence intervals expressed in μM for the tested pesticides Mixture has the same concentration as single test.

a)

%AChE INHIBITION		
Pesticide	**RTG-2**	
	IC20 (μM)	**95% confidence limits**
Chlorpyrifos	*0,14*	*(0,06-0,25)*
Carbofuran	*1,29*	*(0,61- 2,28)*
Mixture	*1,95*	*(1,28-2,79)*

b)

%AChE INHIBITION		
Pesticide	**RTG-2**	
	IC50 (μM)	**95% confidence limits**
Chlorpyrifos	*1,04*	*(0,61-1,76)*
Carbofuran	*31,44*	*(17,65-67,44)*
Mixture	*32,42*	*(22,31-50,63)*

c)

%AChE INHIBITION		
Pesticide	**RTG-2**	
	IC80 (μM)	**95% confidence limits**
Chlorpyrifos	*7,99*	*(4,39-18,06)*
Carbofuran	*767,57*	*(275,21-3708,36)*
Mixture	*538,73*	*(281,22-1267,24)*

We observed that there is relation between cytotoxicity and AChE inhibition. Figure 25 shows acetylcholinesterase inhibition versus % cytotoxicity expressed as percentage to

unexposed control cells after an exposure of 48 h to chlorpyrifos, carbofuran and their mixture.

In the case of chlorpyrifos at lower concentrations (0.1 to 1 μM) while no cytotoxic effect is observed (8.7-16.9%), the AChE inhibition is very important (25.5-49.4%) (Table 1 of the annex 1 of results), showing a sublethal effect (Figure 25). At the maximum concentration tested (100 μM) the cytotoxicity is 58.28% while the AChE inhibition is complet 100%. The AChE inhibition caused by chlorpyrifos is higher than cytotoxicity IC50=1.04 μM versus CI50= 35.94 μM (Table 14).

Otherwise, for carbofuran and the mixture, it seems that AChE inhibition occurs only when cytotoxic effect is observed (Table 14 and Figure 25). At lower concentrations such as 1 μM of carbofuran, little cytotoxicity (23%) and AChE inhibition (26%) is observed. At 1 μM of the mixture a cytotoxicity value of 22.3% was observed and only a 16 % of AChE inhibition. (Table 1 of the annex 1 of results).

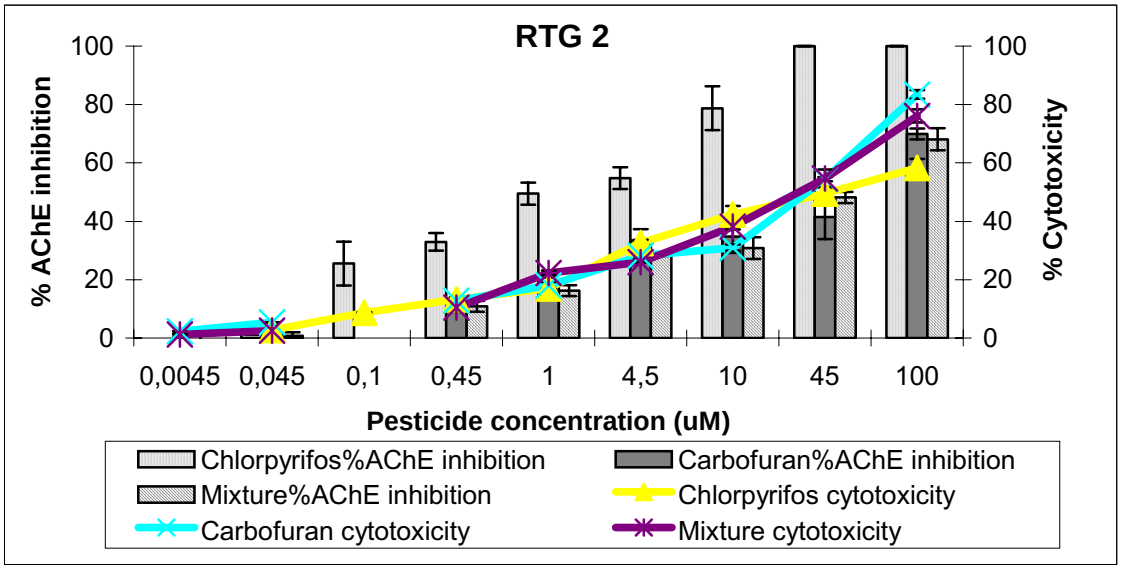


Figure 25. Acetylcholinesterase inhibition of RTG-2 cell line versus cytotoxicity after an exposure time of 48 h to chlorpyrifos, carbofuran and their mixture. Values are the mean of two independent experiments, with 2 enzymatic determinations for AChE inhibition and 6 for cytotoxicity and their SD represented as standard error bars.

4.1.4 Genotoxicity. Comet assay

4.1.4.1. Qualitative analysis

The percentages of DNA comets in RTG-2 cell line cultured for 24 h and then exposed to various concentrations of chlorpyrifos and carbofuran during 48 h were expressed as percentage of unexposed controls and were shown in Figures 26 and 27. At least 75 cells were scored per sample.

A non-cytotoxic concentration range was used for the genotoxicity assay in order to work at lower concentrations than those that produce a cytotoxic effect. By means of the neutral red uptake inhibition assay, Kenacid blue protein assay and ATP determination described above the cytotoxicity of chlorpyrifos, carbofuran and the mixture was evaluated. At 10 nM we observed that there was no cytotoxic effect, so the range tested for both pesticides was 10, 5, 1 nM and 500, 50, 5 and 1 pM.

At 50 pM of chlorpyrifos in RTG-2 cell line the percentage of DNA migration or comets after an exposure of 48 h is about 9% (Figure 26). At 10 nM of chlorpyrifos the percentage is 14%.

At 50 pM of carbofuran the percentage of comets is 5%. But at higher concentrations, such as 10 nM we observed a higher percentage of comets than the observed in chlorpyrifos (17% in front of 14%) (Figure 27)

That indicates that the genotoxic effect of carbofuran is higher than the produced by chlorpyrifos. Moreover, carbofuran is more cytotoxic than chlorpyrifos in RTG-2 cells as stated above.

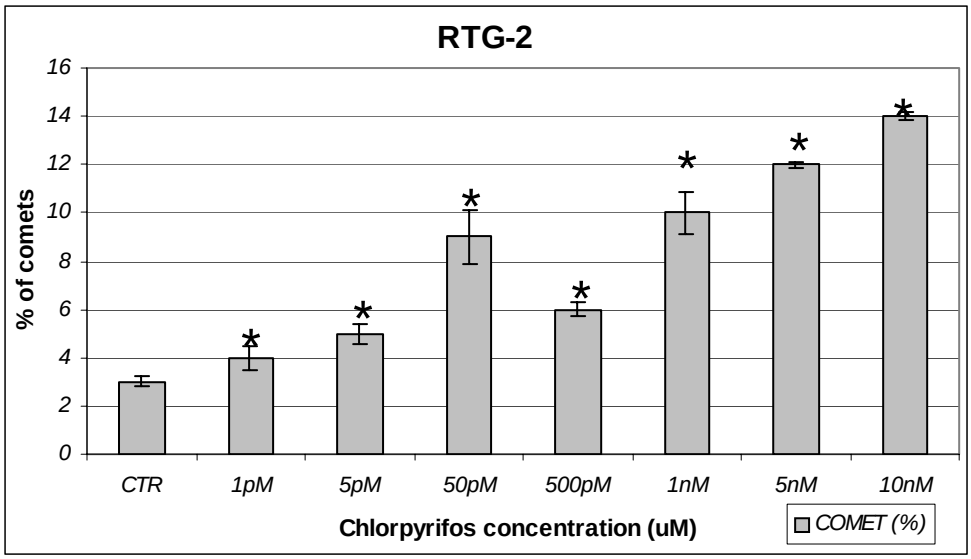


*Figure. 26. Percentage of DNA comets in RTG-2 cell line cultured for 48 h and then exposed to various concentrations of chlorpyrifos expressed as percentage of unexposed controls. Three slides were prepared per assay and 75 nuclei were counted per slide. Bars are means of SD. *= statistically different from control*

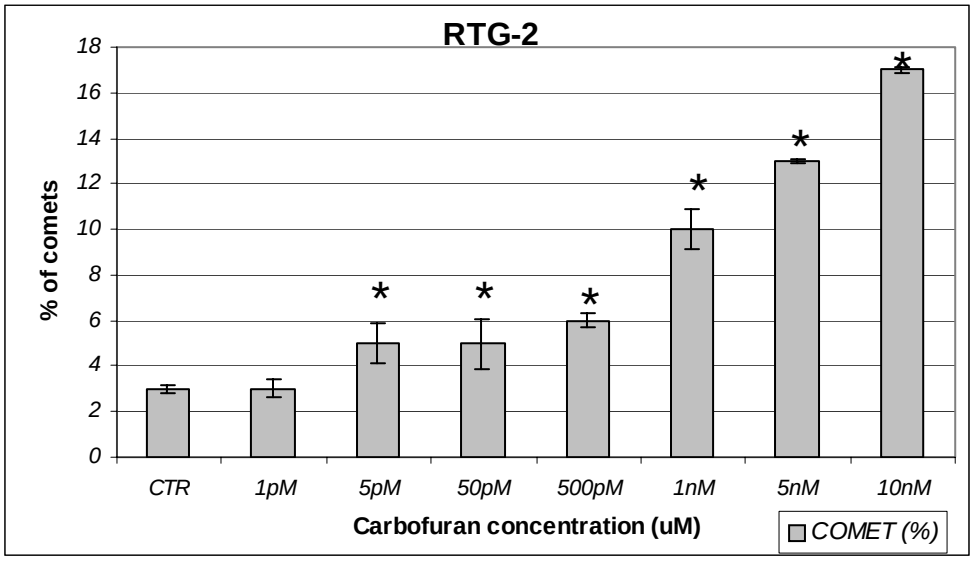


*Figure. 27. Percentage of DNA comets in RTG-2 cell line cultured for 48 h and then exposed to various concentrations of carbofuran expressed as percentage of unexposed controls. Three slides were prepared per assay and 75 nuclei were counted per slide. Bars are means of SD. *= statistically different from control*

4.1.5 Aromatase activity

4.1.5.1. Progesterone (P4) determination

*Progesterone production (functional aromatase activity) expressed as nanograms/ml/nºcell*100 of RTG-2 cell line after exposure time of 48 h to chlorpyrifos and carbofuran is shown in Figure 28 and 29.*

*We observed that RTG-2 cell line presents very low aromatase activity. Progesterone (P4) production is lower than 7 ng/ml/nºcell*100 (Figure 28 and 29). The P4 production is constant although there is an increment of cytotoxicity when pesticide concentrations increase*

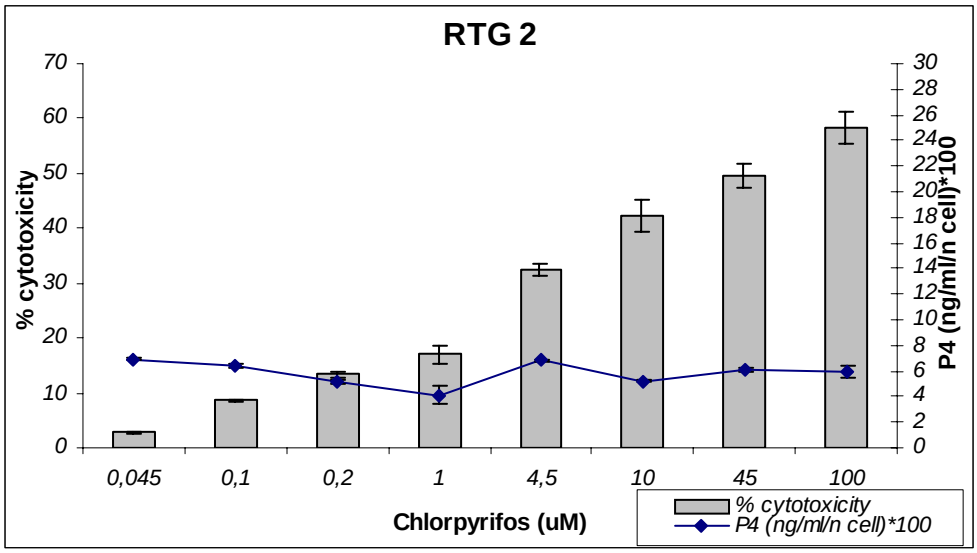


Figure 28. Progesterone (P4) production in RTG-2 cell line expressed in ng P4/ml/nº cells x100 after an exposure period of 48 h to different concentrations of chlorpyrifos (M). Values are presented as the arithmetic mean +/- standard deviation (S.D.) in error bars. Three independent experiments with two replicates each one.

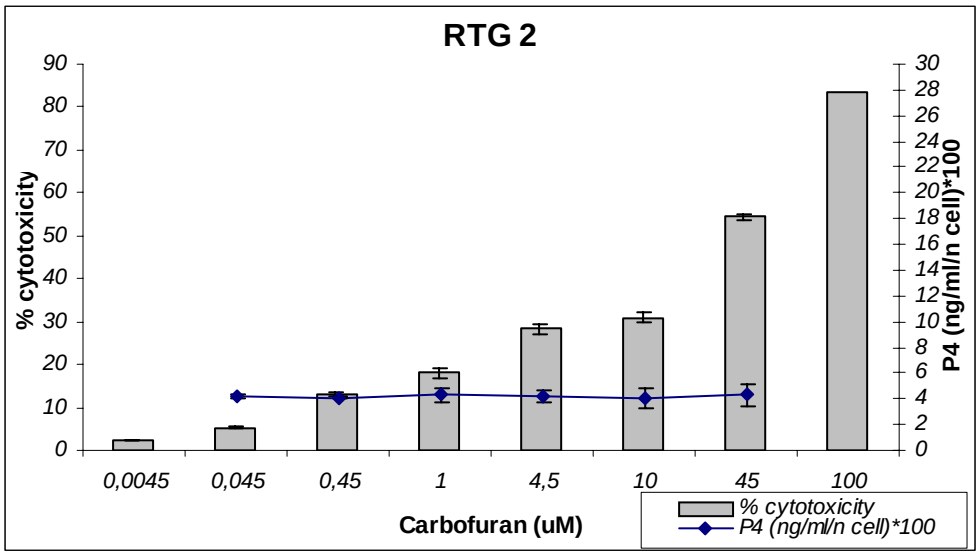


Figure 29. Progesterone (P4) production in RTG-2 cell line expressed in ng P4/ml/nº cells x100 after an exposure period of 48 h to different concentrations of carbofuran. Values are presented as the arithmetic mean +/- standard deviation (S.D.)in error bars. Three independent experiments with two replicates each one

4.2. Bovine granulosa cells

4.2.1. Cytotoxicity

As the in vitro test using RTG-2 cell line, we examined changes in cell morphology of the bovine granulosa cells after an exposure time of 48h to chlorpyrifos, carbofuran and the mixture are represented in Figures 30, 31, 32, respectively. We can observe that in the three exposures, the control cells (unexposed to pesticides and maintained only in MEM) display always a fully confluent monolayer (Figure 30, 31, 32 a). Furthermore, cells exposed to 2.2 μM of chlorpyrifos, 0.45 μM of carbofuran and 0.45 μM of their mixture show a discernible change in the density of the monolayer. There is a little difference respect to control. We only observe a subtle change in the cell density of the monolayer compared to control cells (Figure 30, 31, 32 b). When the concentration of pesticide increases, the morphological changes are bigger compared to control cells. At 10 μM of chlorpyrifos, carbofuran and mixture cells display breaks in the cell monolayer (Figure 30, 31, 32 c). In contrast, cells exposed to 100 μM of chlorpyrifos, carbofuran and their mixture (Figure 30, 31, 32 d) show evident changes in cell shape. At that concentration the maximum effect is observed and cell damage is obvious because the integrity of the cell monolayer is lost.

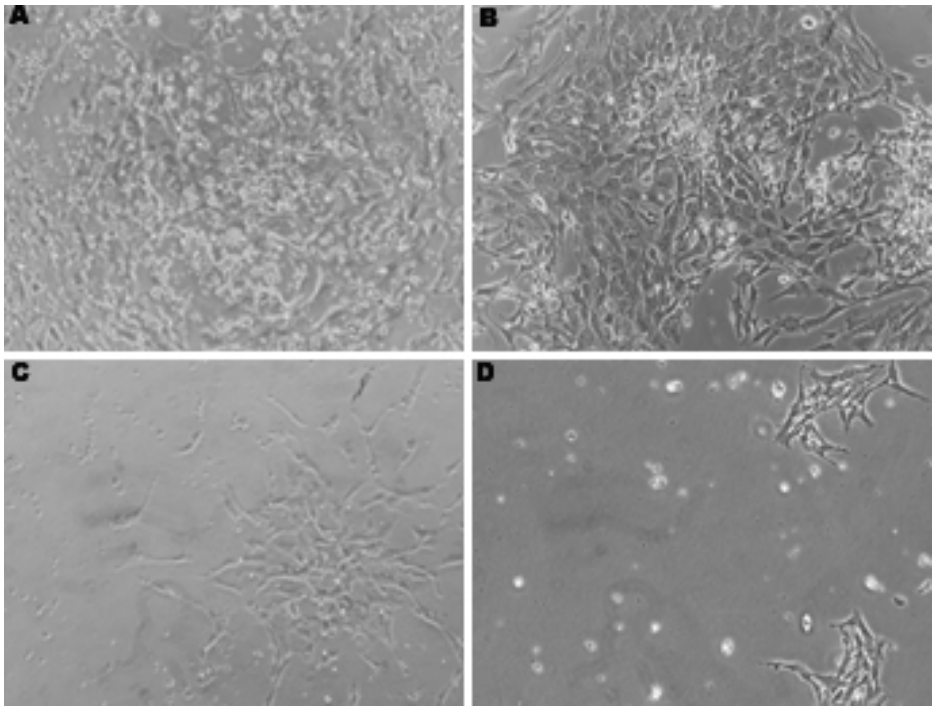


Figure 30. Light microscopy appearance of bovine granulosa cells following exposure to chlorpyrifos: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 2,2 μM of chlorpyrifos showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of chlorpyrifos illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

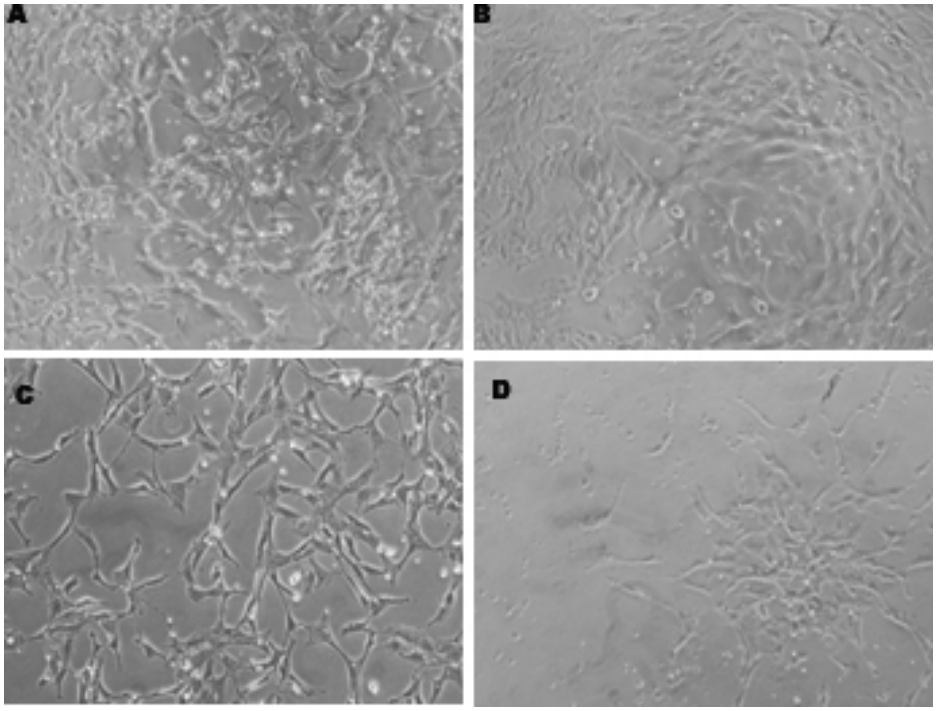


Figure 31. Light microscopy appearance of bovine granulosa cells following exposure to carbofuran: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 0.45 μM of carbofuran showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of cabofuran illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

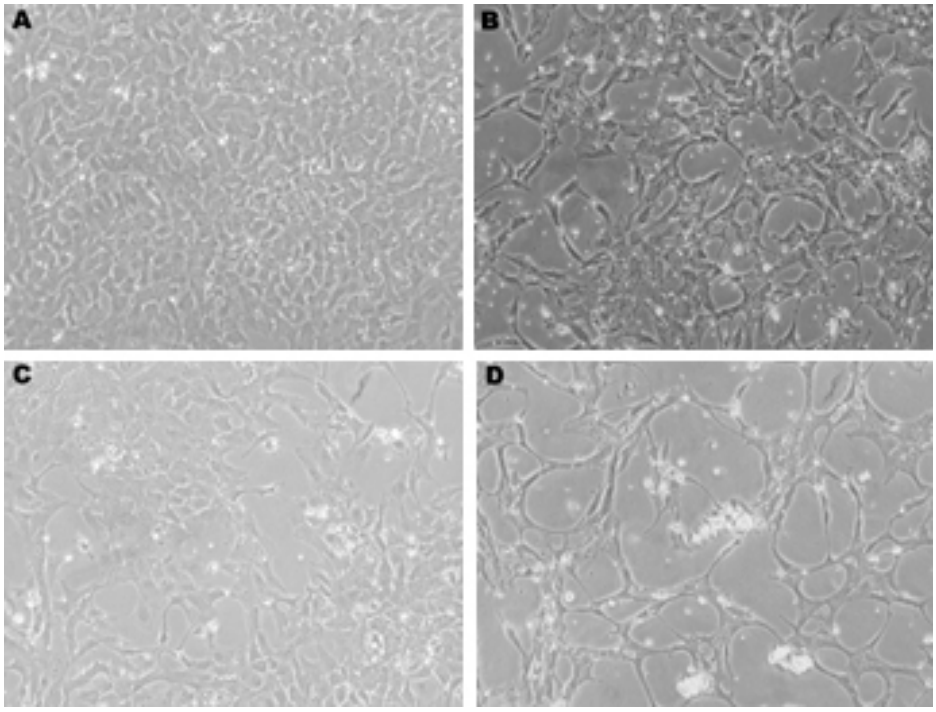


Figure 32. Light microscopy appearance of bovine granulosa cells following exposure to mixture chlorpyrifos-carbofuran: a) control cells maintained in MEM displaying a fully confluent monolayer; b) cells exposed to 0.45 μM of the mixture showing a discernible change in the density of the monolayer; c) cells exposed to 10 μM displaying breaks in the cell monolayer and d) cells exposed to 100 μM of mixture illustrating evident changes in cell shape and clear loss of cell monolayer integrity. Magnification: 10 x

After checking the appearance of bovine granulosa cells by a light microscopy the respective HTD values for chlorpyrifos, carbofuran and the mixture were reported and are shown in Table 5 expressed in μM. As we have stated in RTG-2, the HTD is defined as the highest test pesticide concentration that causes the minimum morphological changes to cells (Shúilleabháin et al, 2004; Liebsch and Spielmann, 1995). As we could appreciate in the microscopic observation (Figure 30, 31, 32 b) the minimum effects start at 2.2 μM of chlorpyrifos, 0.45 μM of carbofuran and 0.45 μM of their mixture. Therefore, carbofuran and the mixture are more toxic than chlorpyrifos because a lower concentration (0.45 μM) is needed to cause the same subtle changes than 2.2 μM of chlorpyrifos.

Table 5. Values of pesticide that causes the minimum morphological changes to bovine granulosa cells after an exposure to 48 h to chlorpyrifos, carbofuran and a mixture chlorpyrifos-carbofuran expressed in μM.

Pesticide	HTD (μM)
Chlorpyrifos	*2.2*
Carbofuran	*0.45*
Mixture	*0.45*

Following HTD evaluation, the cytotoxicity of each pesticide was measured using the same three enpoints than in RTG-2 cells, the Neutral Red assay (NR), the Kenacid Blue Protein assay (KB) and the Adenosin triphospate determination (ATP).
Cell viability was measured after 3 h of incubation to the dye neutral red, the lysosoms of the living cells incorporated the dye and presented the appearance showed in Figure 33.

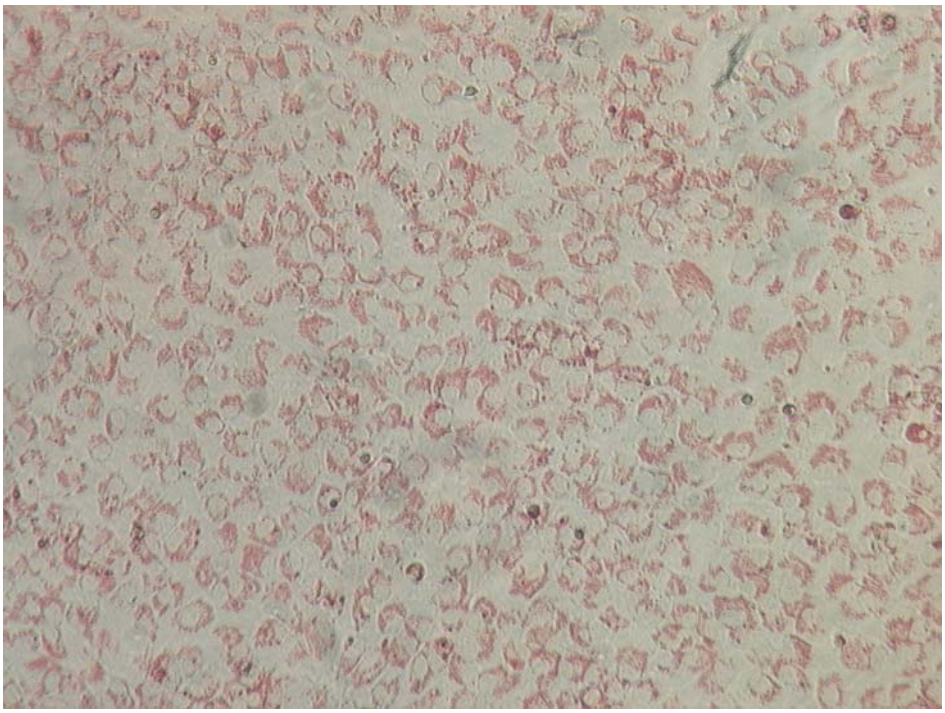


Figure 33. Light microscopic appearance of following 3 h of incubation to neutral red dye. The living cells incorporate the colorant into the lysosoms. Magnification: 10x

The inhibition of cell proliferation was measured by the changes in total cell protein by the Kenacid Blue dye binding method. After 30 min of incubation to the dye Kenacid Blue the cells were stained as shows Figure 34.

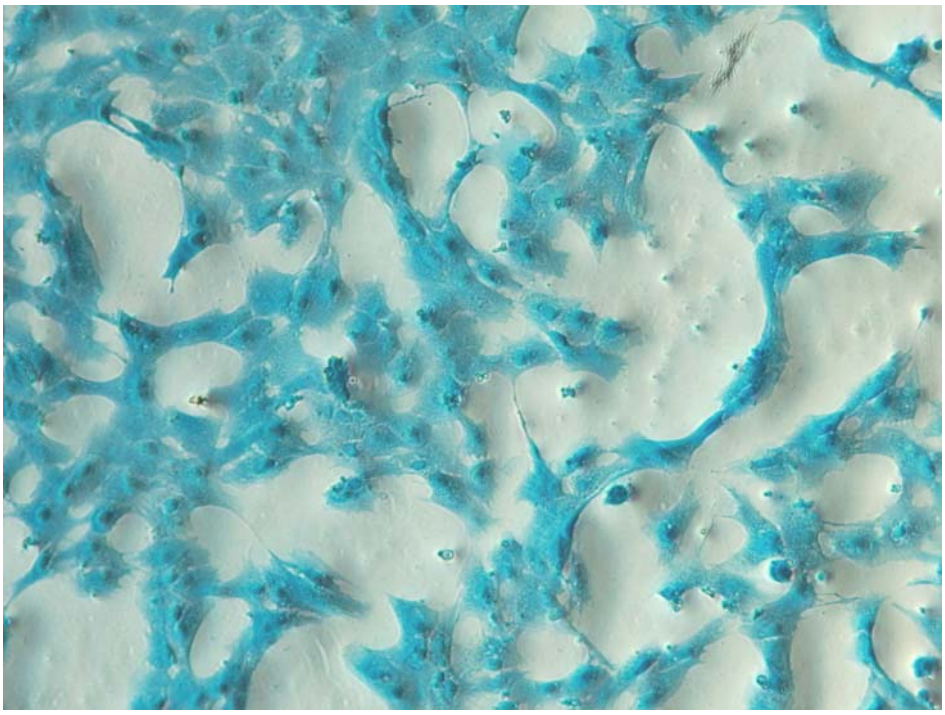


Figure 34. Light microscopic appearance of bovine granulosa cells after 30 min of incubation to Kenacid Blue dye. Magnification: 10 x

The obtained cytotoxicity values using the three endpoints were compared for several concentrations of chlorpyrifos, carbofuran and their mixture and were represented in Figure 35 a-c. Values are expressed as percentage to control cells.
We observe a correlation between these endpoints. In general as occurs in RTG-2 cells, there is a tendency being the neutral red uptake and the ATP content the most sensitive endpoints and protein determination the less sensitive, but there are no statistical differences.

a)

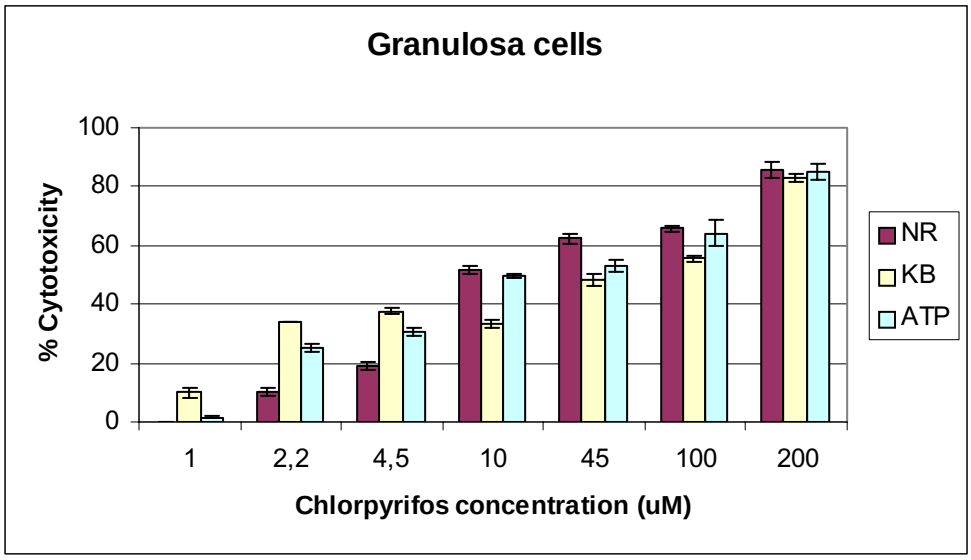


Figure 35-a. Cytotoxicity of chlorpyrifos to bovine granulosa cells as determined by 48h NR, KB, ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments

b)

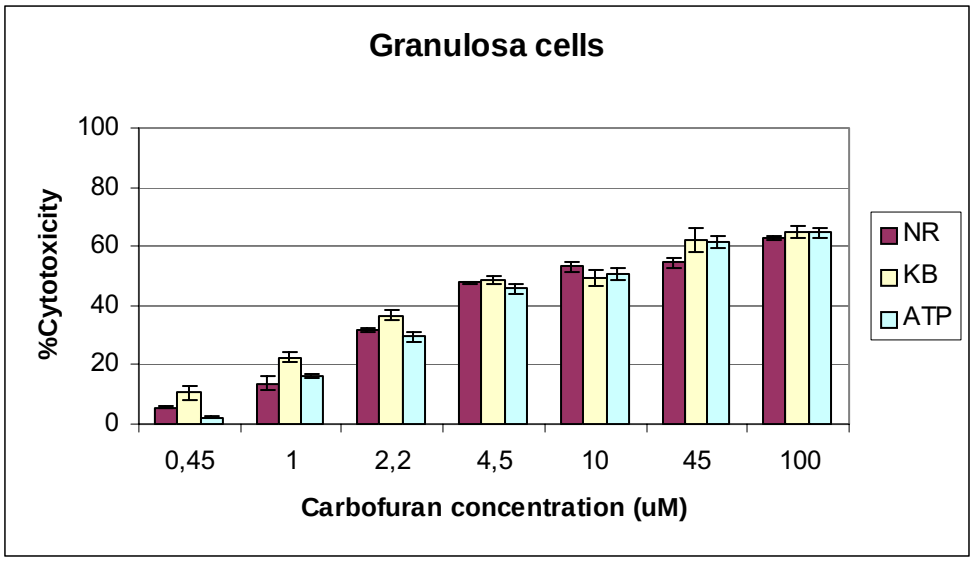


Figure 35-b. Cytotoxicity of carbofuran to bovine granulosa cells as determined by 48h NR, KB, ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments.

c)

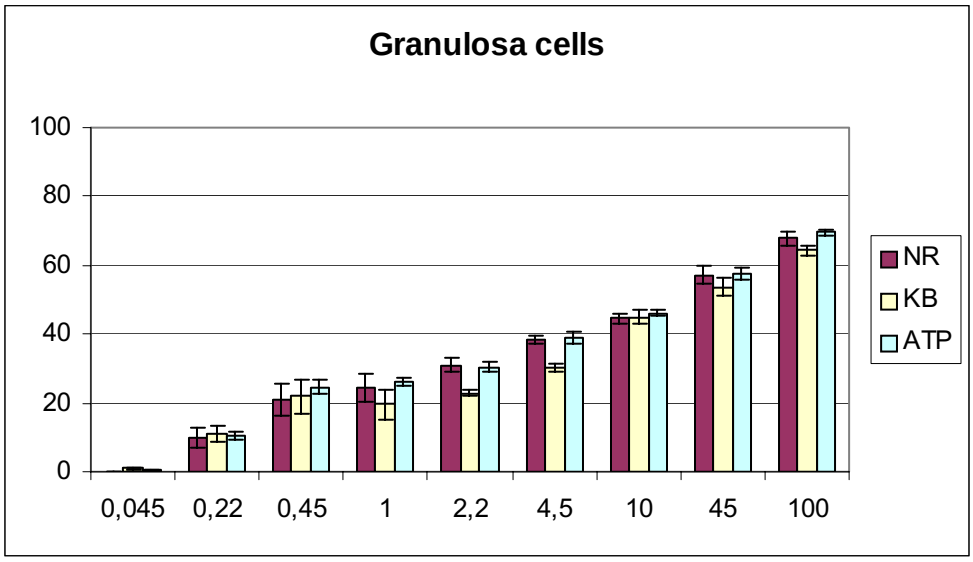


Figure 35-c. Cytotoxicity of mixture chlorpyrifos-carbofuran to bovine granulosa cells as determined by 48h NR, KB, ATP. Data expressed as percentage of unexposed controls +/- SD of two independent experiments. Mixture values are the sum of the concentration of chlorpyrifos and carbofuran in test solutions (concentration chlorpyrifos=concentration carbofuran).

The percentages of cytotoxicity in bovine granulosa cells related to the used pesticide concentrations after 48 h of exposure are shown in Table 2 of the annex 2 of results. Table 6 shows the obtained EC20, EC50, EC80 values and its 95% confidence intervals expressed in M for the tested pesticides using the three endpoints, Neutral red uptake (NR), Kenacid blue protein (KB) and ATP measurement. In order to integrate the three endpoints in a single data, a cytotoxicity index (CI) was determined by the equation of Castaño et al., 1994b, and it is also reported in Table 6. According to values presented in Table 6, carbofuran seems more toxic than chlorpyrifos (there are statistical differences at 20% but not at 50 and 80%). The concentration that causes 20% of cytotoxicity according to the cytotoxicity index (CI) is 3.41 μM for chlorpyrifos, 1.03 μM for carbofuran and 1.10 μM for the mixture. The concentration that causes the 50% of cytotoxicity is 36.2 μM for chlorpyrifos, 23.05 μM for carbofuran and 21.33 μM for the mixture. The concentration that causes the 80% of cytotoxicity is 383 μM for chlorpyrifos, 516 μM for carbofuran and 412 μM for the mixture.

Table 6. a) EC20, b)EC50, c)EC80 and its 95% confidence intervals expressed in μM *for the tested pesticides using the in vitro endpoints and the Castaño cytotoxicity index. NR=Neutral Red, KB= Kenacid Blue, ATP= Adenosin triphospate. CI= Cytotoxicity index. Mixture has the same concentration as single test. Each pesticide was tested in a separate plate to avoid interferences between compounds. Two plates for each pesticide were performed with six replicates for NR and KB and two for ATP.*

a)

GRANULOSA CELLS								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC20 (μM)	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**
Chlorpyrifos	*2,30*	*(0,85-4,31)*	*1,40999*	*(0,39-3,02)*	*2,47*	*(1,09-4,31)*	*3,41*	*(2,12-5,00)*
Carbofuran	*1,80*	*(1,02-2,86)*	*1,29884*	*(0,75-2,03)*	*1,93*	*(1,18-2,92)*	*1,03*	*(0,43-1,85)*
Mixture	*,81*	*(0,54-1,15)*	*1,04291*	*(0,68-1,49)*	*,69*	*(0,45-0,99)*	*1,10*	*(0,75-1,54)*

b)

GRANULOSA CELLS								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC50 (μM)	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**
Chlorpyrifos	*16,16*	*(9,53-27,86)*	*26,87*	*(15,28-53,56)*	*22,27*	*(14,30-36,31)*	*36,20*	*(24,08-61,55)*
Carbofuran	*17,37*	*(10,81-31,50)*	*13,53*	*(8,69-23,14)*	*15,90*	*(10,60-26,30)*	*23,05*	*(14,03-45,04)*
Mixture	*16,24*	*(11,64-23,93)*	*24,57*	*(16,75-39,10)*	*14,64*	*(10,47-21,62)*	*21,33*	*(15,07-32,21)*

c)

GRANULOSA CELLS								
Pesticide	***NR***		***KB***		***ATP***		**CI**	
	EC80 (μM)	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**
Chlorpyrifos	*113,65*	*(59,00-324,60)*	*511,96*	*(192,32-2964,70)*	*200,43*	*(104,54-545,84)*	*383,89*	*(186,91-1107,17)*
Carbofuran	*168,01*	*(79,81-500,08)*	*140,87*	*(70,12-378,86)*	*132,26*	*(70,15-321,35)*	*516,27*	*(193,14-2613,10)*
Mixture	*323,60*	*(175,88-711,25)*	*578,71*	*(284,38-1479,13)*	*309,93*	*(167,38-687,97)*	*412,08*	*(193,14-2613,10)*

4.2.2. Acetylcholinesterase activity staining

Acetylcholinesterase (AchE) activity of bovine granulosa cells was stained according to the method of Karnovsky et al. (1964).

Three treatments were done as in RTG-2 cell line; results were reported in Table 7.

Treatment 1= Staining solution,

Treatment 2= Staining solution + substrate acetylthiocholine (AcSCh),

Treatment 3= Eserine hemisulfate (an inhibitor of the esterases) was added to the staining solution + AcSCh in order to observe a possible degradation of the substrate AcSCh by other sources different to esterases.

Table 7. Acetylcholinesterase activity staining in bovine granulosa cells.

Acetylcholinesterase activity staining		
Treatment 1	Staining solution	No colour observed
Treatment 2	Staining solution+AcSCh	Colour observed
Treatment 3	Staining solution+ AcSCh +eserine hemisulfate	No colour observed

The appearance of the cells was assessed by light microscopy. Figure 36-a, which corresponds with treatment 1 shows the control cells, there is no colour observed due to the absence of substrate AcSCh.

In contrast, in the treatment 2 (Figure 36-b), there is a formation of a brown precipitate that shows AChE activity.

In treatment 3 (Figure 36-c), with presence of eserine hemisulfate, there is not formation of the brown precipitate indicating that all the colour produced in treatment 2 is due to the activity of esterases.

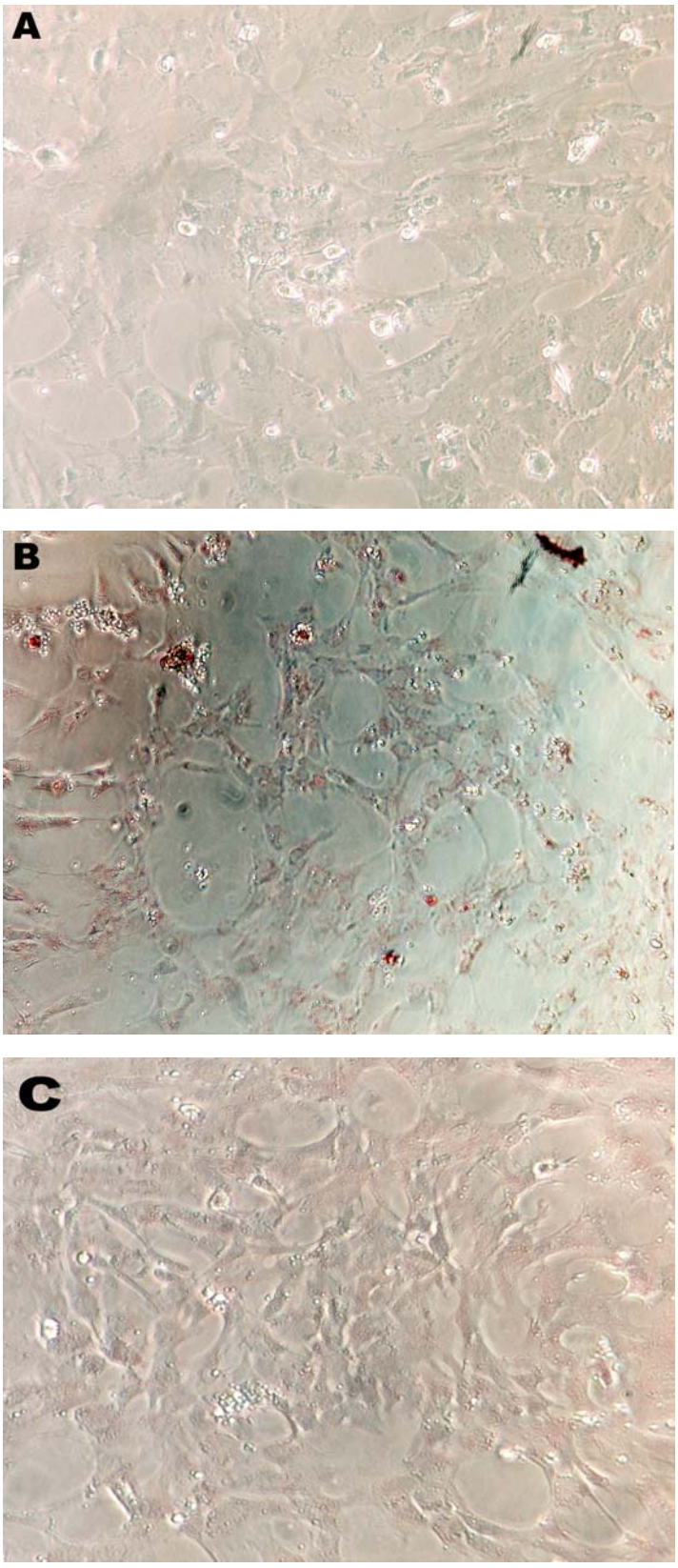


Figure 36. Acetylcholinesterase activity in bovine granulosa cells: a) Treatment 1: control cells presented negative staining because there was no substrate AcSCh (acetylthiocholine); b) Treatment 2: cells presented positive staining, there is the formation of a brown precipitate; c) Treatment 3: cells presented negative staining due to the effect of eserine hemisulfate, an inhibitior of the esterases. Magnification: 40 x

4.2.3. Acetylcholinesterase assay

4.2.3.1. Enzyme characterisation

4.2.3.1.1. Substrate specificity

In order to optimize the AChE activity measurements in granulosa cells three substrates, acetylthiocholine (AcSCh), Propionylthiocholine (PrSCh) and Butyrylthiocholine (BuSCh) were assayed as in RTG-2.

Relative enzyme activities in bovine granulosa cells expressed in nmol/min/mg protein as function of increasing concentrations of AcSCh, PrSCh and BuSCh expressed in mM is showed in Figure 37.

The highest activity was found with AcSCh (3.2 mM). A low activity was measured for PrSCh (1.94 mM) and BuSCh (1.47 mM). The specifity of the enzyme for the substrate AcSCh suggests that the enzyme is acetylcholinesterase (AChE).

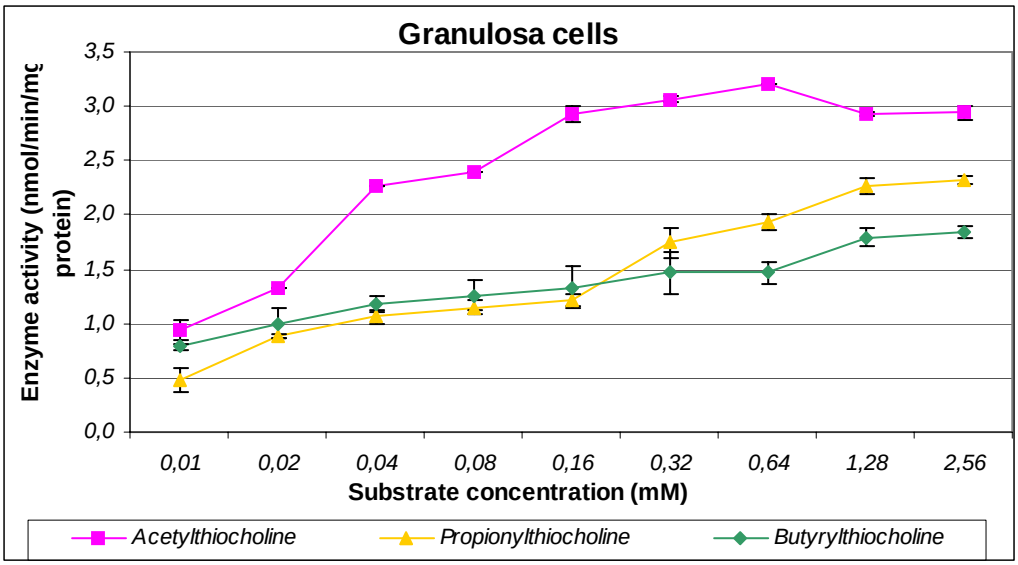


Figure 37. Enzyme activity of bovine granulosa cells expressed as nmol/min/mg prot as function of AcSCh, PrSCh and BuSCh concentration expressed in mM. Values are the mean of two independent experiments with two enzymatic determinations for AChE an three for proteins and corresponding standard error bars.

4.2.3.1.2. Enzyme inhibition

Effects of different inhibitors: eserine hemisulfate, iso-OMPA and BW284C51 on enzyme actitivity of bovine granulosa cells are showed in Figures 38, 39, 40.

Eserine hemisulfate, an inhibitor of all the esterases, significantly inhibited enzyme activity in bovine granulosa cells (Figure 38) after an incubation period of 45 min. Inhibition by eserine hemisulfate is almost complete (85%) at the highest concentration tested (0.1 mM). BW284C51, a specific inhibitor of acetylcholinesterases, also caused a high inhibition (79.06%) after an incubation period of 45 min at concentration of 0.1 mM (Figure 40). In contrast, bovine granulosa cells were relatively insensitive to iso-OMPA, an inhibitor of butyrylcholinesterases, since it only causes a 40% inhibition. It suggests that the main enzyme present is acetylcholinesterase.

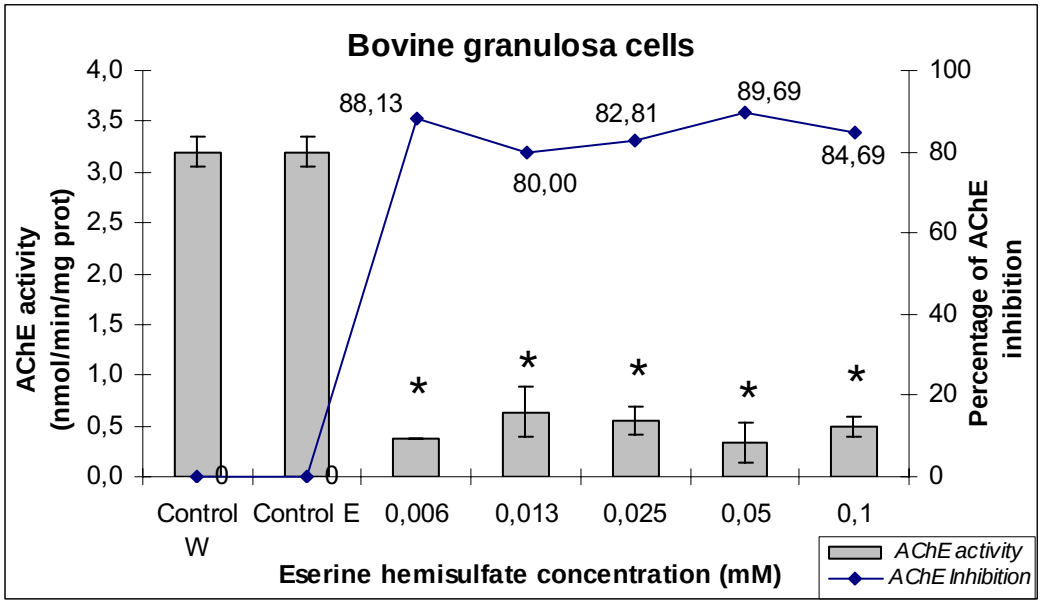


*Figure 38. Effects of eserine hemisulfate (expressed in mM) on enzyme actitivity (expressed in nmol/min/mg prot) of bovine granulosa cells after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

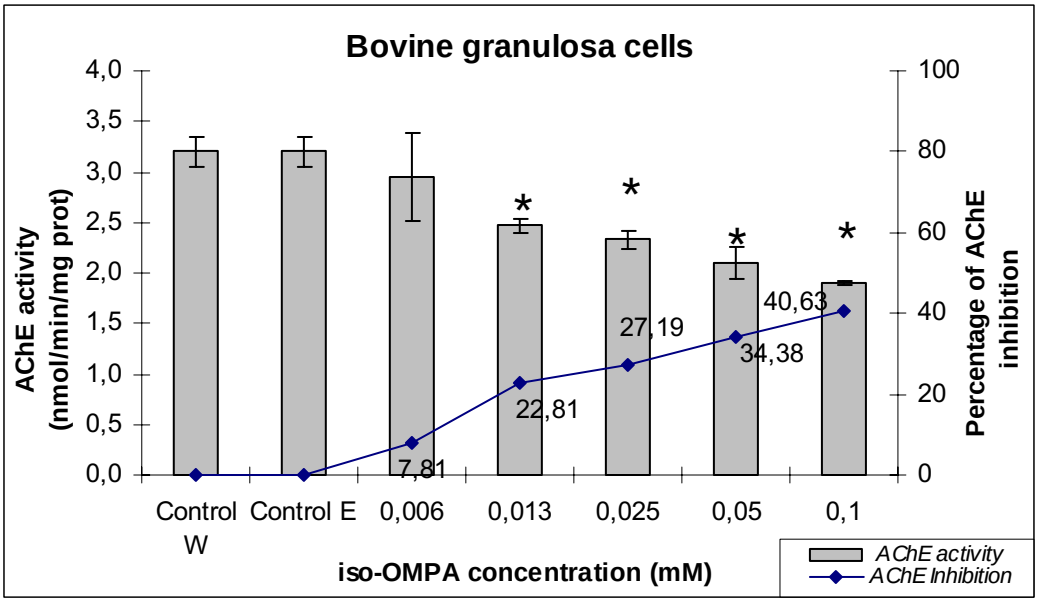


*Figure 39. Effects of iso-OMPA (expressed in mM) on enzyme actitivity expressed in (nmol/min/mg prot) of bovine granulosa cells after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

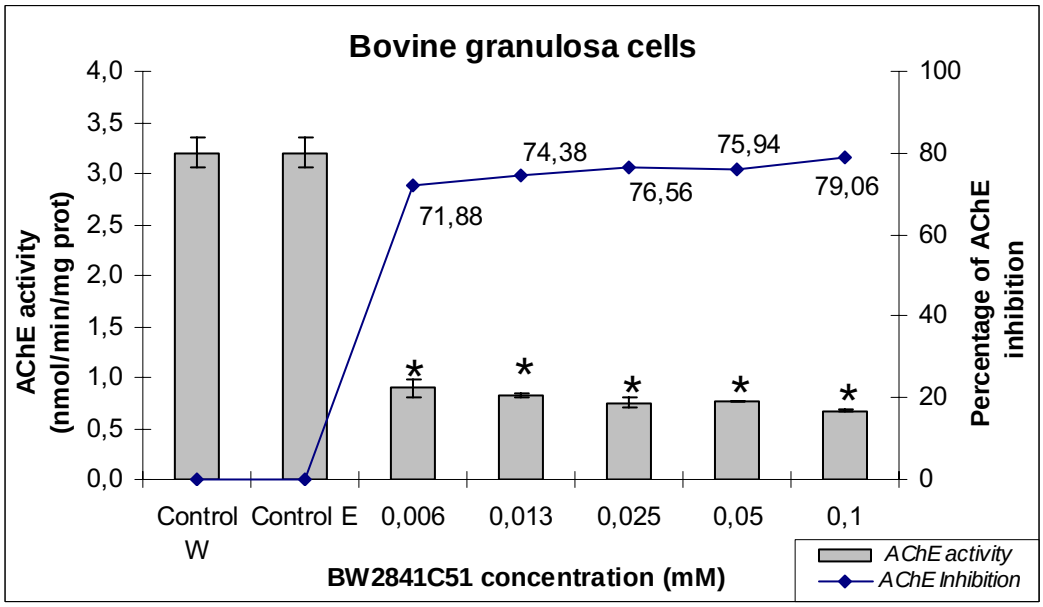


*Figure 40. Effects of BW284C51 (expressed in mM) on enzyme actitivity expressed in (nmol/min/mg prot) of bovine granulosa cells after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

4.2.3.1.3 Optimisation of AChE determination

Effect of substrate concentration on esterase activity expressed in nmol/min/mg protein with acetylthiocholine as substrate in bovine granulosa cells and the Michaelis-Menten constant (Km) is shown in Figure 2 of the annex 2 of results.

AChE activity of bovine granulosa cells shows an apparent Michaelian behaviour. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot). The apparent Km value for the substrate AcSCh is 21.7 μM. The maximum rate of hydrolysis, Vmax, was achieved at 3153 μM (Figure 2 from the annex 2 of results). Inhibition of enzymatic activity was observed at the highest concentration of AcSCh tested, 2.56 mM (Figure 2 from the annex 2 of results).

4.2.3.2. Enzyme determination

The percentages of AChE inhibition in bovine granulosa cells related to the used pesticide concentrations after 48 h of exposure are shown in Table 2 of the annex 2 of results. Table 8 shows acetylcholinesterase inhibition values of IC20, IC50, IC80 and its 95% confidence intervals expressed in μM for the tested pesticides. Chlorpyrifos inhibits more than carbofuran the acetylcholinesterase activity. Although the concentration that causes an inhibition of the 20% (IC20) is 1.91 μM for chlorpyrifos, 0.61 μM for carbofuran and 2.7 μM for the mixture, the concentration that causes the 50% of the enzyme activity (IC50) is 20.96 μM for chlorpyrifos, whereas it is 53.78 μM for carbofuran and 54.47 μM for the mixture.

The IC80 values are 229.96 μM for chlorpyrifos, 4713 μM for carbofuran and 1100 μM for the mixture.

Table 8. Acetylcholinesterase inhibition of bovine granulosa cells a) IC20, b) IC50, c) IC80 and its 95% confidence intervals expressed in μM for the tested pesticides Mixture has the same concentration as single test.

a)

%AChE INHIBITION		
Pesticide	**Granulosa Cells**	
	IC20 (μM)	**95% confidence limits**
Chlorpyrifos	*1,91*	*(0,27-4,85)*
Carbofuran	*0,61*	*(0,40-0,88)*
Mixture	*2,70*	*(1,85-3,79)*

b)

%AChE INHIBITION		
Pesticide	**Granulosa Cells**	
	IC50 (μM)	**95% confidence limits**
Chlorpyrifos	*20,96*	*(9,20-58,63)*
Carbofuran	*53,78*	*(33,51-96,86)*
Mixture	*54,47*	*(34,97-95,61)*

c)

%AChE INHIBITION		
Pesticide	**Granulosa Cells**	
	IC80 (μM)	**95% confidence limits**
Chlorpyrifos	*229,96*	*(76,07-2903,11)*
Carbofuran	*4713,84*	-
Mixture	*1100,24*	*(498,53-3206,57)*

Acetylcholinesterase inhibition of bovine granulosa cells versus % cytotoxicity after an exposure of 48h to chlorpyrifos, carbofuran and their mixture is shown in Figure 41

Like occurs in RTG-2, there is relation between cytotoxicity and AChE inhibition. Figure 41 shows acetylcholinesterase inhibition versus % cytotoxicity expressed as percentage to unexposed control cells after an exposure of 48 h to chlorpyrifos, carbofuran and their mixture.

In the case of chlorpyrifos at lower concentrations (0.22 to 1 μM) while no cytotoxic effect is observed (0-4.39%), the AChE inhibition is important (15.88-26.91%) (Table 2 of the annex 2 of results), showing a sublethal effect (Figure 41). At the maximum concentration tested (200 μM) the cytotoxicity is 59.89% while the AChE inhibition is almost complet 95.58%. The AChE inhibition caused by chlorpyrifos is higher than cytotoxicity IC50=20.96 μM versus CI50= 36.2 μM (Table 14).

Furthermore, for carbofuran, at lower concentrations (0.1 to 2.2 μM) there is no cytotoxic effect (0-16.73%) but AChE inhibition occurs (12.35-24.6%). At the maximum concentration (100 μM) the effect is the same 54.7 % cytotoxicity and 51.47 % AChE inhibition. In contrast, for the mixture the cytotoxicity is higher than inhibition of acetylcholinesterase. At the maximum concentration (100 μM) the percentage of observed cytotoxicity is 71.51% in front of an AChE inhibition of 55.9%. (Table 2 from the annex 2 of results). The CI50 is 21.23 μM whereas the IC50 is 54.47 μM.

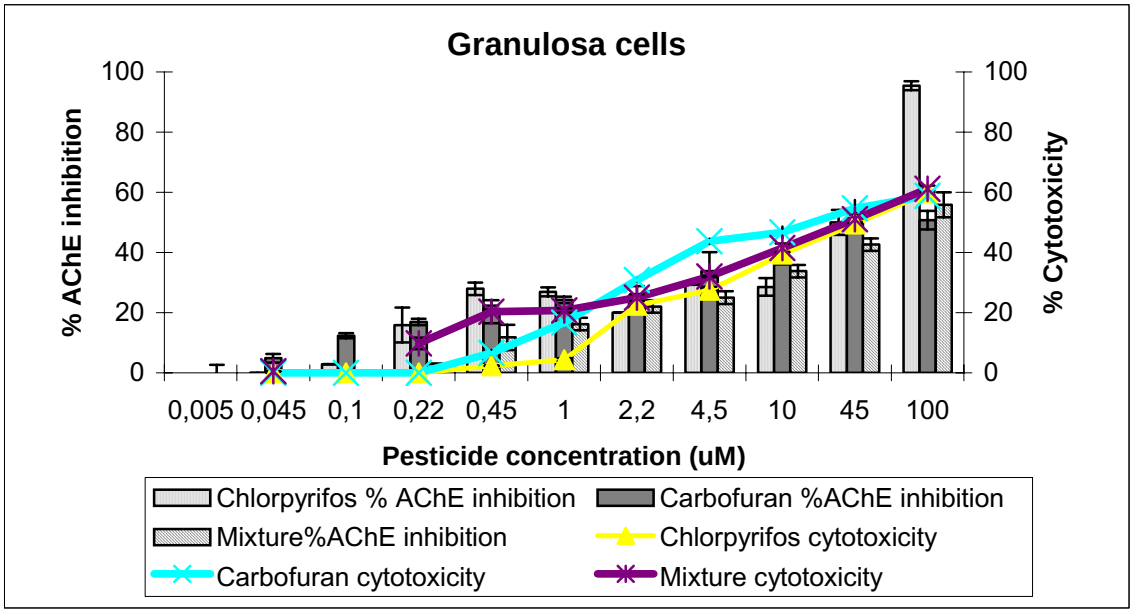


Figure 41. Acetylcholinesterase inhibition of bovine granulosa cells versus cytotoxicity after an exposure time of 48 h to chlorpyrifos, carbofuran and their mixture. Values are the mean of two independent experiments, with 2 enzymatic determinations for AChE inhibition and 6 for cytotoxicity and their SD represented as standard error bars.

4.2.4. Genotoxicity. Comet assay

4.2.4.1. Qualitative analysis

The percentages of DNA comets in bovine granulosa cells cultured for 24 h and then exposed to various concentrations of chlorpyrifos and carbofuran during 48 h were expressed as percentage of unexposed controls and were shown in Figures 42 and 43. At least 75 cells were scored per sample.

A non-cytotoxic concentration range was used for the genotoxicity assay in order to work at lower concentrations than those that produce a cytotoxic effect. By means of the neutral red uptake inhibition assay, Kenacid blue protein assay and ATP determination described above the cytotoxicity of chlorpyrifos, carbofuran and the mixture was evaluated. At 5 nM for chlorpyrifos and 1 nM for carbofuran we observed that there was no cytotoxic effect, so the range tested was 5, 1 nM and 500, 50, 5 and 1 pM for chlorpyrifos and 1 nM, 500, 50, 5 and 1 pM for carbofuran.

At 50 pM of chlorpyrifos in bovine granulosa cells the percentage of DNA migration or comets after an exposure of 48 h is 3.2% (Figure 42). At 1 nM the percentage is 8.6. At 5 nM of chlorpyrifos, maximum concentration, the percentage is 14%.

At 50 pM of carbofuran the percentage of comets is 2.1% after 48 h of exposure. At the higher concentration 1 nM, we observed a higher percentage of comets than the observed for chlorpyrifos (12% in front of 8.6%) (Figure 43)

That indicates that the genotoxic effect of carbofuran is higher than the produced by chlorpyrifos. Moreover, carbofuran is more cytotoxic than chlorpyrifos in bovine granulosa cells as stated above.

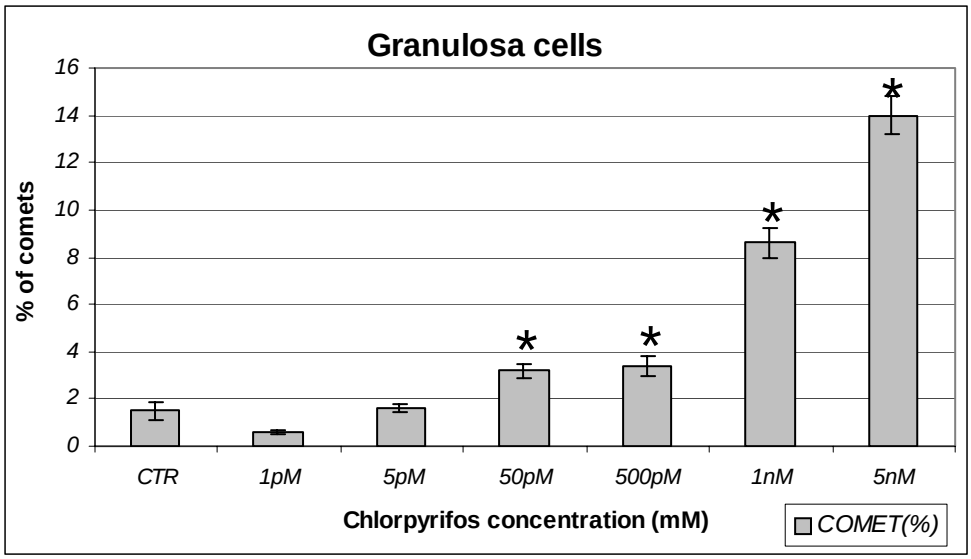


Figure. 42 Percentage of DNA comets in granulosa cells cultured for 48 h and then exposed to various concentrations of chlorpyrifos expressed as percentage of unexposed controls. Three slides were prepared per assay and 75 nuclei were counted per slide. Bars are means of SD. *= statistically different from control

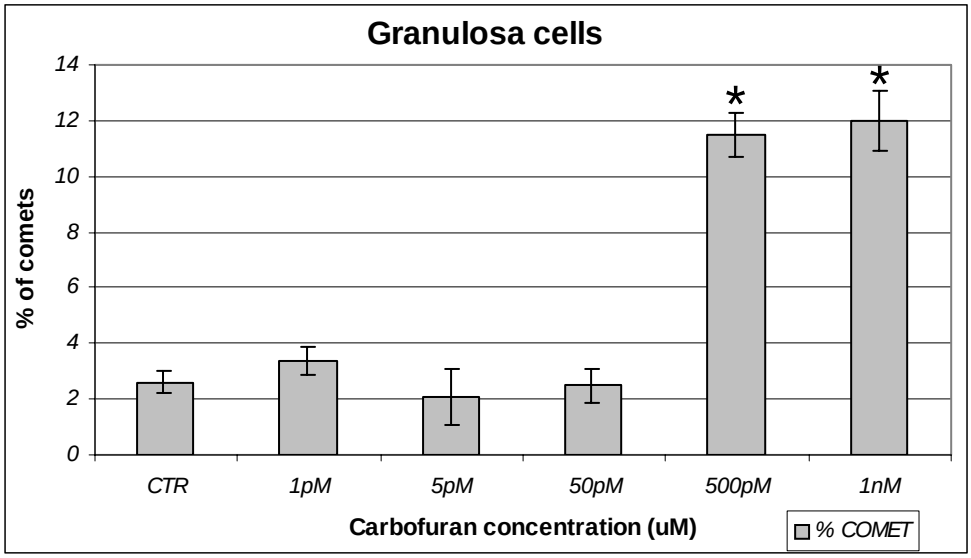


Figure 43. Percentage of DNA comets in granulosa cells cultured for 48 h and then exposed to various concentrations of carbofuran expressed as percentage of unexposed controls. Three slides were prepared per assay and 75 nuclei were counted per slide. Bars are means of SD. *= statistically different from control

4.2.5. Aromatase activity

4.2.5.1. Progesterone (P4) determination

Progesterone production (functional aromatase activity) of bovine granulosa cells after exposure time of 48 h to chlorpyrifos and carbofuran is shown in Figure 44 and 45.

Progesterone production (functional aromatase activity) expressed as nanograms/ml/nºcell in bovine granulosa cells after exposure time of 48 h to chlorpyrifos and carbofuran is shown in Figure 42 and 43.

We observed that bovine granulosa cells present high aromatase activity. Progesterone (P4) production is about 18-36 ng/ml/nºcell and tends to increase with the highest toxic concentrations (Figure 44 and 45).

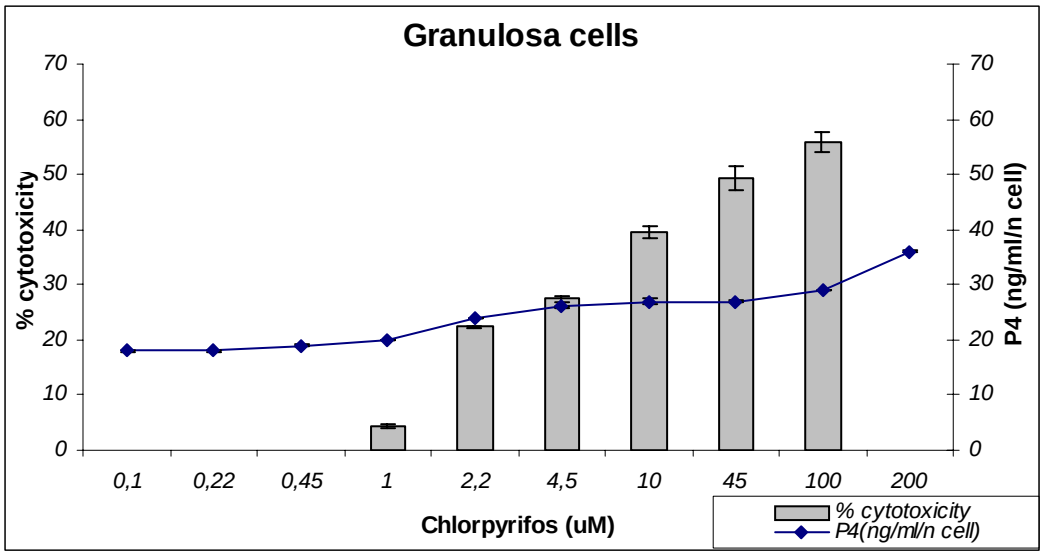


Figure 44. Progesterone (P4) production in bovine granulosa cells expressed in ng P4/ml/nº cells after an exposure period of 48 h to different concentrations of chlorpyrifos. Values are presented as the arithmetic mean +/- standard deviation (S.D.) Three independent experiments with two replicates each one.

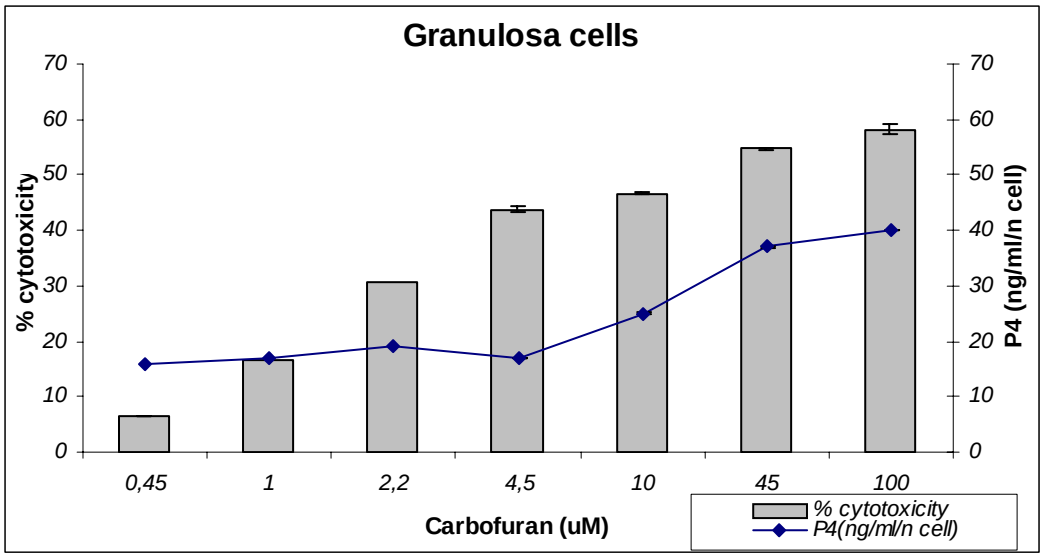


Figure 45. Progesterone (P4) production in bovine granulosa cells expressed in ng P4/ml/nº cells after an exposure period of 48 h to different concentrations of carbofuran. Values are presented as the arithmetic mean +/- standard deviation (S.D.) Three independent experiments with two replicates each one

In vivo results

4.3. *Danio rerio*

4.3.1. Acute toxicity assay

The percentages of mortality in Danio rerio related to the used pesticide concentrations after 96 h of exposure are shown in Table 3 of the annex 3 of results. The values of acute toxicity EC20, EC50, EC80 and its 95% confidence intervals expressed in μM are shown in Table 9. According to values presented in this table, carbofuran is more toxic than chlorpyrifos. The concentration that causes 20% of mortality is 36.91 μM for chlorpyrifos, 16.78 μM for carbofuran and 12.19 μM for the mixture. The concentration that causes the 50% of mortality is 72.71 μM for chlorpyrifos, 28.88 μM for carbofuran and 32.45 μM for the mixture. The concentration that causes the 80% of mortality is 143 μM for chlorpyrifos, 49.74 μM for carbofuran and 86.36 μM for the mixture.

Table 9. Acute toxicity values of EC20, EC50, EC80 and its 95% confidence intervals expressed in μM

Pesticide	% Fish mortality					
	LC20 (μM)	95% confidence limits	LC50 (μM)	95% confidence limits	LC80 (μM)	95% confidence limits
Chlorpyrifos	36,91	(23,02-48,21)	72,71	(57,83-87,99)	143,23	(115,44-202,06)
Carbofuran	16,78	(12,43-20,44)	28,88	(24,35-33,54)	49,74	(42,31-62,04)
Mixture	12,19	(6,80-17,25	32,45	(23,29-52,48)	86,36	(53,19-239,58)

Moreover, the behavioral responses of Danio rerio were observed at 1–8 h and every 24 h during the acute toxicity tests. The control group consists of unexposed fish to pesticides. It showed normal behavior during all the test period. In contrast, in presence of different concentrations of pesticides the changes in behavioral response started 1 h after dosing. At lower concentrations of chlorpyrifos the behaviour was similar with the control group. At 0.24 μM showed less general activity with occasional loss of equilibrium that becomes more frequent in the 4.5 μM concentration. The 45 μM and 100 μM concentration groups stayed motionless close to the water surface and later fell to the aquarium bottom in an uncontrolled manner. The highest concentration of 200 μM showed all responses at high intensities, loss of equilibrium, hanging vertically in water, rapid gill movement, erratic swimming, sudden swimming motion in a spiral

fashion, after long periods of motionlessness lying down on the aquarium bottom and suddenly starting to move.

The exposed fish to carbofuran suffered a shock after 3 h and swimmed as if they were exhausted. At 4.5 μM of carbofuran they showed lethargic movements, although in the following days they survived. At the maximum concentration tested, 100 μM, fishes showed all responses as happened for chlorpyrifos.

With regard to the mixture chlorpyrifos-carbofuran, at lower concentrations like 4.5 μM fishes present less general activity with occasional loss of equilibrium. At 21 μM concentration fishes stayed motionless close to the water surface and later fell to the aquarium bottom in an uncontrolled manner. At 45 μM, the highest concentration, fishes also showed the same observed responses than when chlorpyrifos and carbofuran were tested single.

4.3.2. Acetylcholinesterase assay

4.3.2.1. Enzyme characterisation

4.3.2.1.1. Substrate specificity

Relative enzyme activities of different tissues, head, muscle and liver of the fish Danio rerio were determined with three substrates, Acetylthiocholine (AcSCh), Propionylthiocholine (PrSCh) and Butyrylthiocholine (BuSCh) in order to optimize the AChE activity measurements.

Enzyme activity expressed in nmol/min/mg protein versus function of increasing concentrations of AcSCh, PrSCh and BuSCh expressed in mM is showed in Figures 46, 47 and 48.

In head, the highest activity was found with AcSCh (178.2 mM). A low activity was measured for PrSCh (43.46 mM) and very low activity for BuSCh (3.27 mM). The specifity of the enzyme for the substrate AcSCh suggests that the main enzyme present in head is acetylcholinesterase (AChE).

Similar results were found in muscle, the highest activity was found with AcSCh (577.03 mM). For PrSCh a low activity was found (133.75 mM) and very low activity for BuSCh (2.80 mM). The specifity of the enzyme for the substrate AcSCh suggests that the main enzyme present in muscle is acetylcholinesterase (AChE).

In liver, the highest activity was also found with AcSCh (18.87 mM). A low activity was measured for PrSCh (3.48 mM) and very low activity for BuSCh (1.01 mM). The

specifity of the enzyme for the substrate AcSCh suggests that the main enzyme present in liver is acetylcholinesterase (AChE).

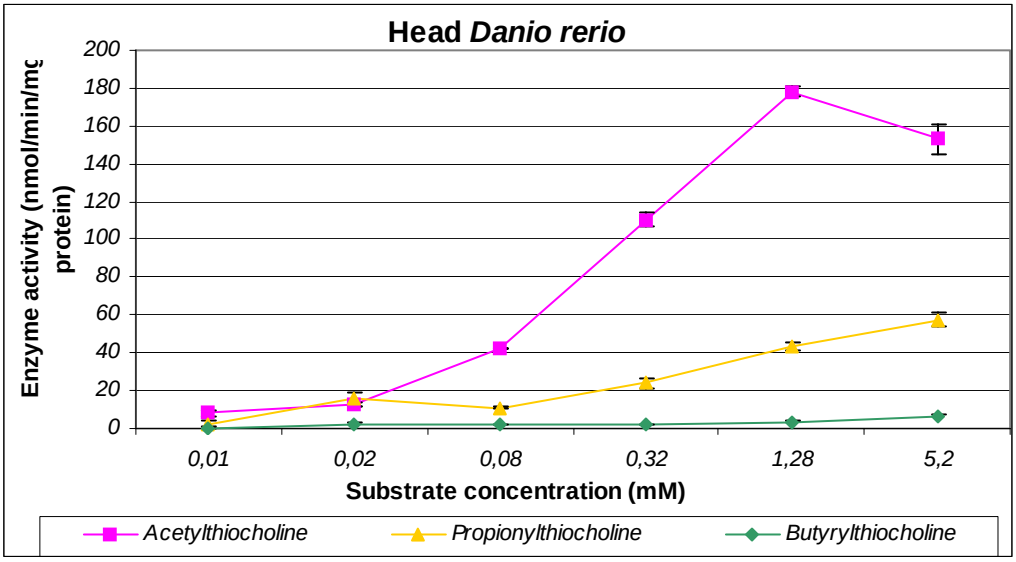


Figure 46. Enzyme activity of head expressed as nmol/min/mg prot of Danio rerio as function of AcSCh, PrSCh and BuSCh concentration expressed in mM. Values are the mean of three independent experiments with two enzymatic determinations for AChE an three for proteins and corresponding standard error bars.

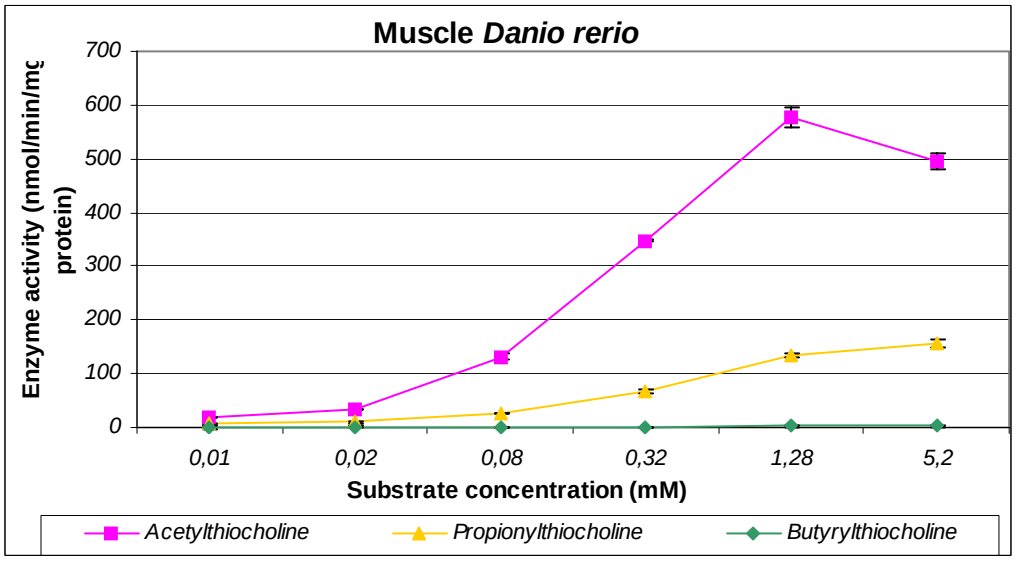


Figure 47. Enzyme activity of muscle expressed as nmol/min/mg prot of Danio rerio as function of AcSCh, PrSCh and BuSCh concentration in mM. Values are the mean of three independent experiments with two enzymatic determinations for AChE an three for proteins and corresponding standard error bars.

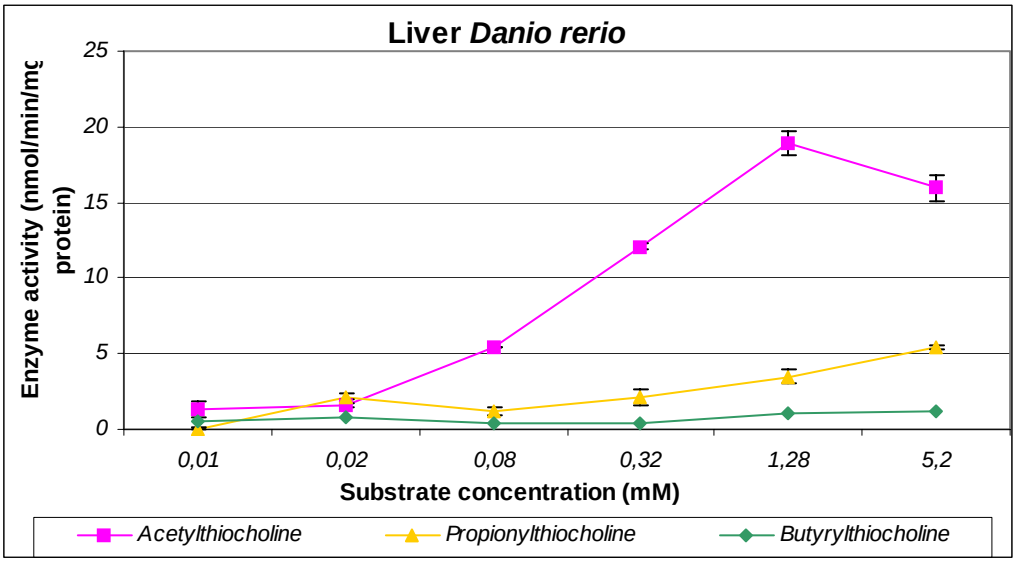


Figure 48. Enzyme activity of liver expressed as nmol/min/mg prot of Danio rerio as function of AcSCh, PrSCh and BuSCh concentration in mM. Values are the mean of three independent experiments with two enzymatic determinations for AChE an three for proteins and corresponding standard error bars.

4.3.2.1.2. Enzyme inhibition

Effects of three inhibitors eserine hemisulfate, iso-OMPA and BW284C51 on enzyme actitivity of head, muscle and liver from Danio rerio are showed in Figures 49 to 57.

Eserine hemisulfate, an inhibitor of all the esterases, significantly inhibited enzyme activity in head, muscle and liver (Figure 49, 52 and 55, respectively) after an incubation period of 45 min. Inhibition by eserine hemisulfate is almost complete 97.72% in head, 98.83% in muscle and 95.74% in liver at the highest concentration tested (0.1 mM). BW284C51, a specific inhibitor of acetylcholinesterases, also caused a high inhibition 99.9% in head, muscle and 99.5% in liver after an incubation period of 45 min at concentration of 0.1 mM (Figure 51, 54 and 57, respectively). On the other hand, the three tissues were relatively insensitive to iso-OMPA, an inhibitor of butyrylcholinesterases, since it only causes a inhibition of 29.73% in head, 16.05% in muscle and 15.45% in liver at the maximum concentration tested (0.1mM) (Figures 50, 53 and 56, respectively). It suggests that the main enzyme present in these tissues is acetylcholinesterase.

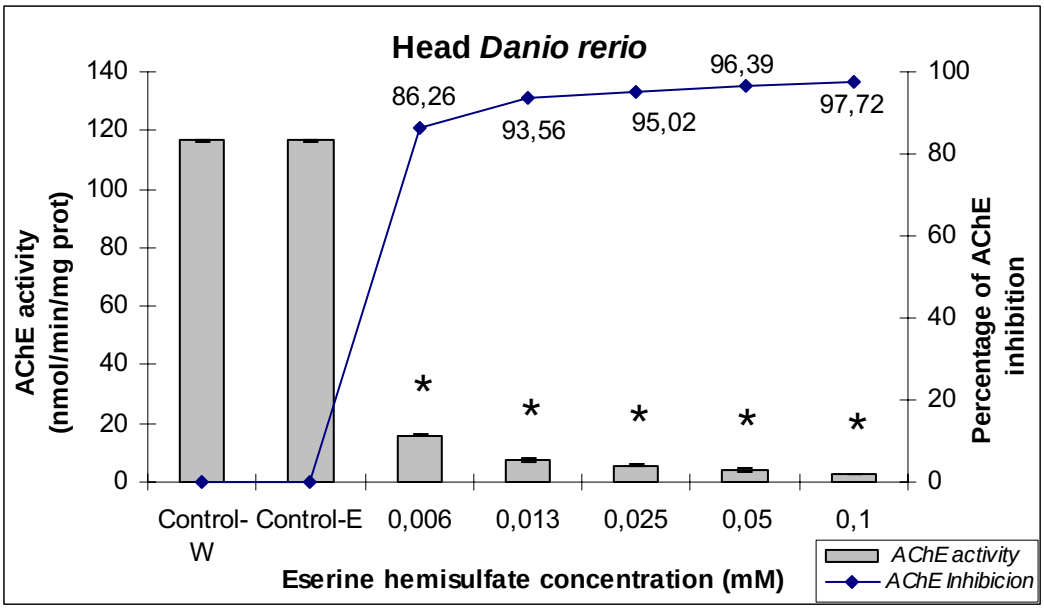


*Figure 49. Effects of eserine hemisulfate (expressed in mM) on enzyme actitivity of head (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control. Columns express AChE activity (nmol/min/mg prot) and the line express the percentage of inhibition.*

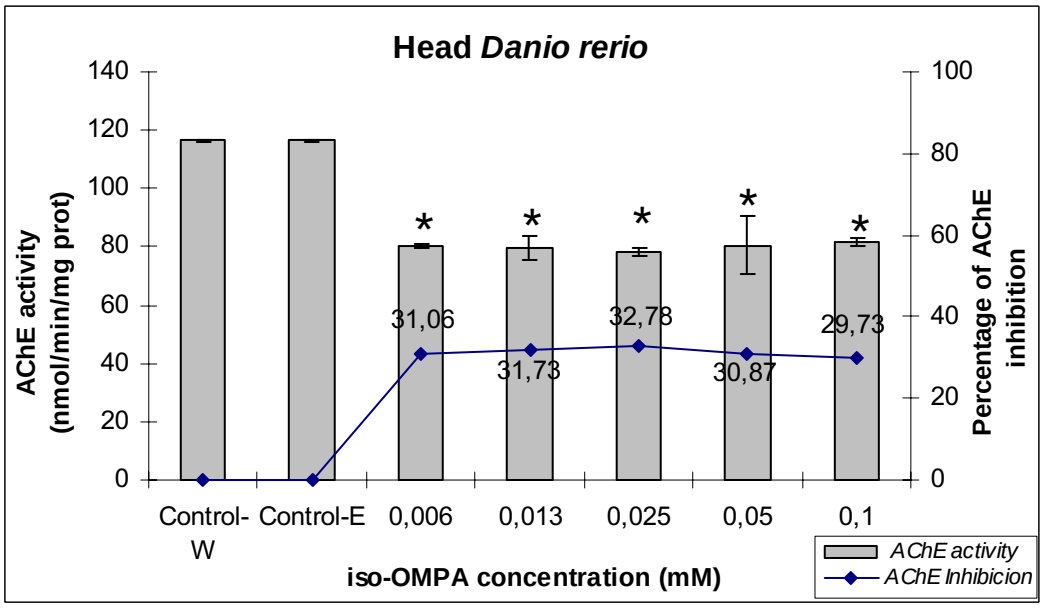


*Figure 50. Effects of iso-OMPA (expressed in mM) on enzyme actitivity of head (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

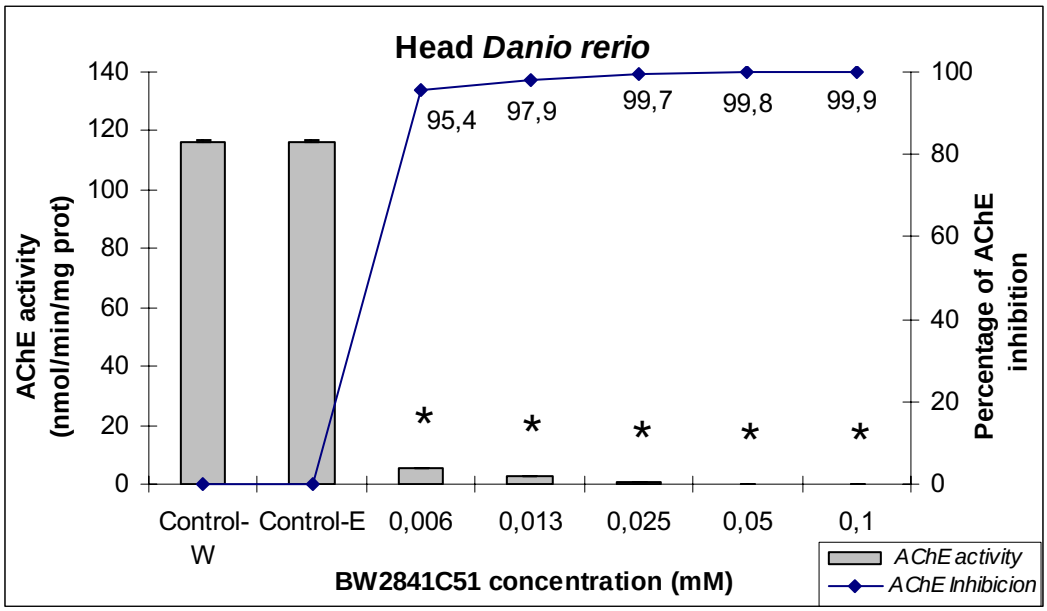


*Figure 51. Effects of BW284C51 (expressed in mM) on enzyme actitivity of head (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

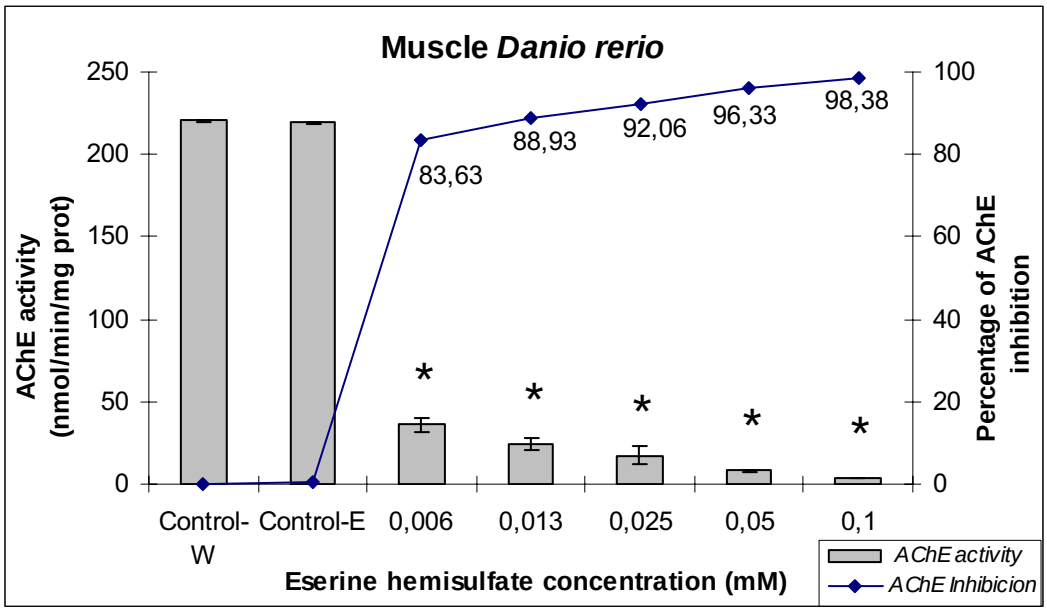


*Figure 52. Effects of eserine hemisulfate (expressed in mM) on enzyme actitivity of muscle (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

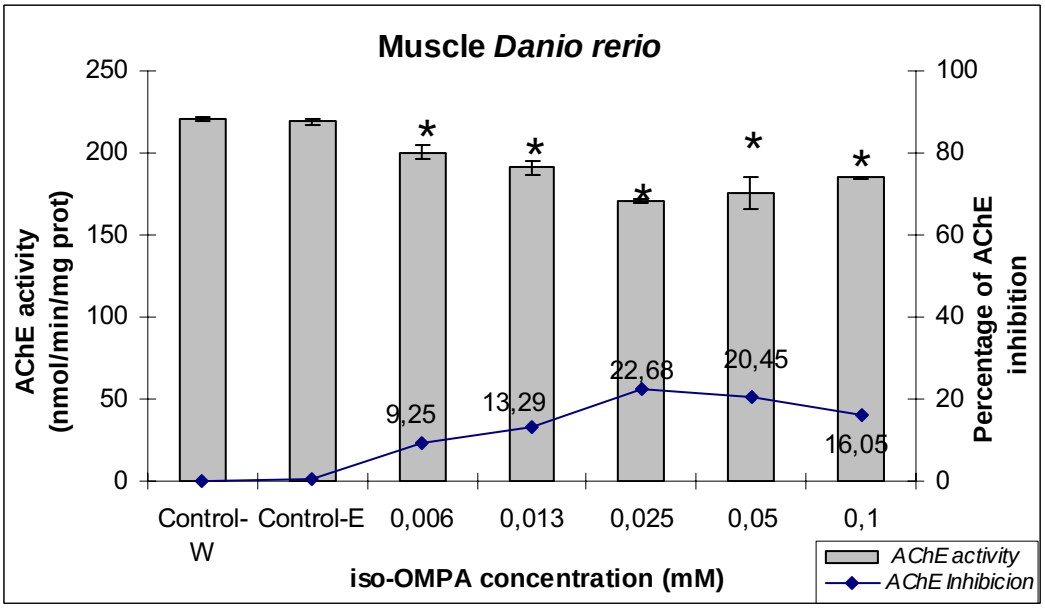


*Figure 53. Effects of iso-OMPA (expressed in mM) on enzyme actitivity of muscle (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

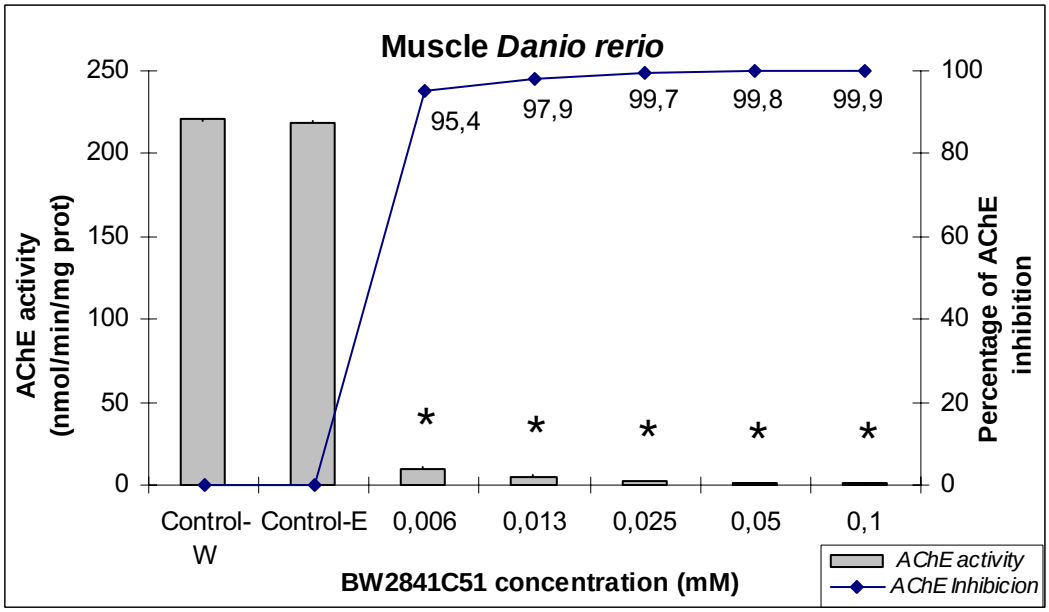


*Figure 54. Effects of BW284C51 (expressed in mM) on enzyme actitivity of muscle (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

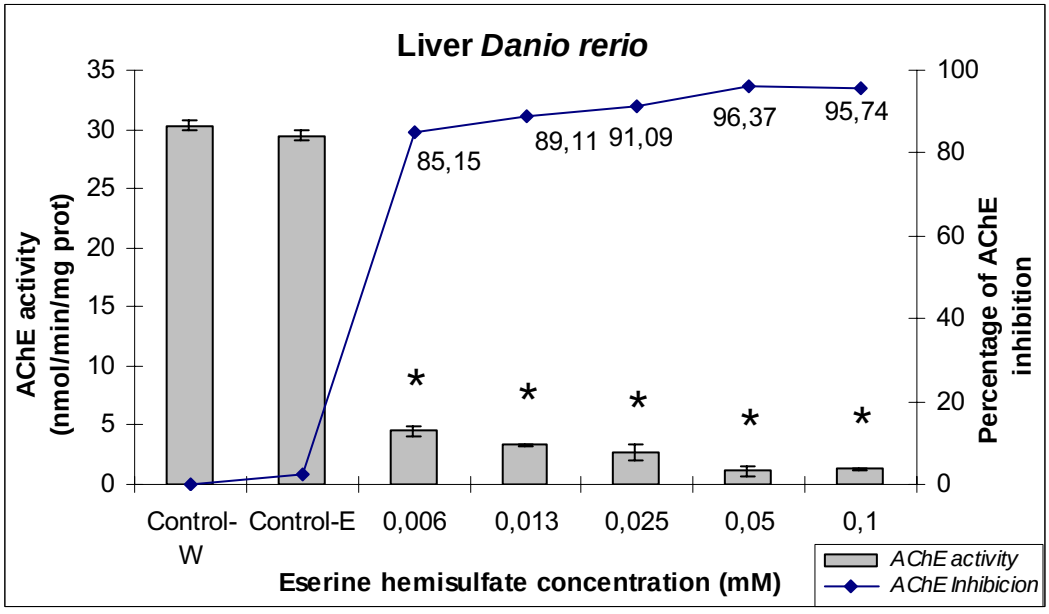


*Figure 55. Effects of eserine hemisulfate (expressed in mM) on enzyme actitivity of liver (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

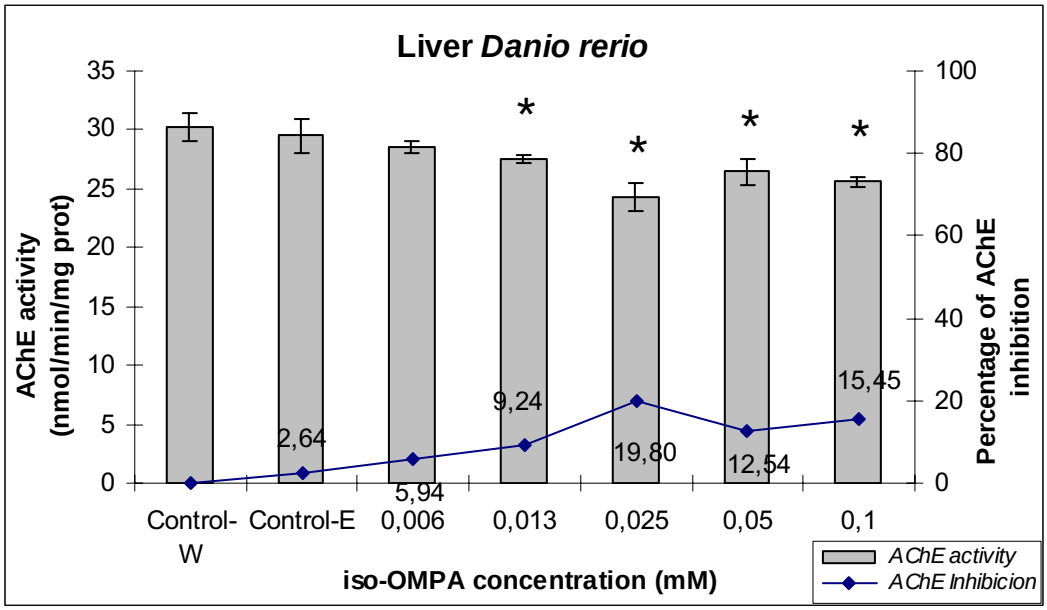


*Figure 56. Effects of iso-OMPA (expressed in mM) on enzyme actitivity of liver (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

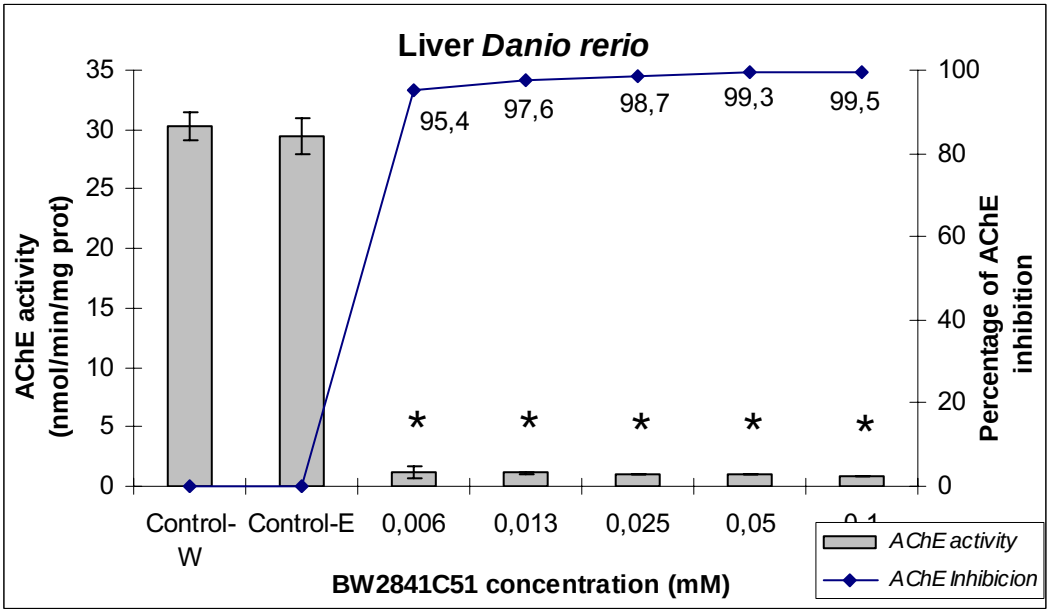


*Figure 57. Effects of BW284C51 (expressed in mM) on enzyme actitivity of liver (expressed in nmol/min/mg prot) of Danio rerio after an incubation period of 45 min at 26ºC. CTR-W=distilled water control; CTR-E=ethanol control, *= statistically different from control.*

4.3.2.1.3ØOptimisation of AChE determination

Effects of substrate concentration on esterase activity expressed in nmol/min/mg protein with acetylthiocholine as substrate in head, muscle and liver of Danio rerio and the Michaelis-Menten constant (Km) are shown in Figure 3, 4 and 5, respectively, of the annex 3 of results.

AChE activity of head, muscle and liver of Danio rerio shows an apparent Michaelian behaviour. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot). The apparent Km value for the substrate AcSCh in head is 0.198 mM, 0.211 mM in muscle and 0.165 mM in liver. The maximum rate of hydrolysis, Vmax, was achieved at 178 mM in head, 577 mM in muscle and 18.5 mM in liver (Figure 3, 4 and 5 from the annex 3 of results).
Inhibition of enzymatic activity was observed at the highest concentration of AcSCh tested, 5.2 mM (Figure 3, 4 and 5 from the annex 3 of results).

4.3.2.2. Enzyme determination

The percentages of AChE inhibition in Danio rerio related to the used pesticide concentrations after 96 h of exposure are shown Table 3 of the annex 3 of results. The concentrations that cause an inhibition of 20, 50 and 80% for acetylcholinesterase activity and its 95% confidence intervals expressed in μM for the tested pesticides in the different tissues (head, muscle and liver) of Danio rerio are shown in Table 10.

Chlorpyrifos inhibits the acetylcholinesterase activity more than carbofuran in the three tissues. In head, the concentration that causes an inhibition of the 20% (IC20) is 0.06 μM for chlorpyrifos, 20.62 μM for carbofuran and 8.16 μM for the mixture, the concentration that causes an inhibition of 50% of the enzyme activity (IC50) is 0.81 μM for chlorpyrifos, whereas it is 43.23 μM for carbofuran and 22.72 μM for the mixture.
The IC80 values are 10.14 μM for chlorpyrifos, 90.62 μM for carbofuran and 63.24 μM for the mixture.
Similar results were obtained in muscle, IC20 is 0.13 μM for chlorpyrifos, 26.09 μM for carbofuran and >45 μM for the mixture, the concentration that causes the 50% of the enzyme activity (IC50) is 1.53 μM for chlorpyrifos, whereas it is 47.65 μM for carbofuran and also >45 μM for the mixture.

The IC80 values are 18.47 μM for chlorpyrifos, 87.01 μM for carbofuran and also >45 μM for the mixture.

In liver, the concentration that causes an inhibition of the 20% (IC20) is 0.21 μM for chlorpyrifos, 22.39 μM for carbofuran and 12.19 μM for the mixture, the concentration that causes an inhibition of 50% of the enzyme activity (IC50) is 2.71 μM for chlorpyrifos, whereas it is 57.34 μM for carbofuran and 32.45 μM for the mixture.

The IC80 values are 34.31 μM for chlorpyrifos, 146.82 μM for carbofuran and 86.36 μM for the mixture.

Table 10. Acetylcholinesterase inhibition in head, muscle and liver of Danio rerio a) IC20, b)IC50, c)IC80 and its 95% confidence intervals expressed in μM for the tested pesticides. Mixture has the same concentration as single test.

a)

%AChE INHIBITION						
Pesticide	***Head***		***Muscle***		***Liver***	
	IC20 (μM)	**95% confidence limits**	**IC20 (μM)**	**95% confidence limits**	**IC20 (μM)**	**95% confidence limits**
Chlorpyrifos	0,06	(0,01-0,24)	0,13	(0,016-0,44)	0,21	(0,05- 0,55)
Carbofuran	20,62	(17,50-23,38)	26,09	(16,9-32,36)	22,39	(12,46- 32,65)
Mixture	8,16	(4,57-11,61)	>45		12,19	(6,80-17,25)

b)

%AChE INHIBITION						
Pesticide	***Head***		***Muscle***		***Liver***	
	IC50 (μM)	**95% confidence limits**	**IC50 (μM)**	**95% confidence limits**	**IC50 (μM)**	**95% confidence limits**
Chlorpyrifos	0,81	(0,20-2,59)	1,53	(0,48-4,64)	2,710	(1,161-6,03)
Carbofuran	43,23	(38,20-50,77)	47,65	(38,15-78,77)	57,34	(38,18-167,73)
Mixture	22,72	(16,59-32,70)	>45	-	32,45	(23,30-52,48)

c)

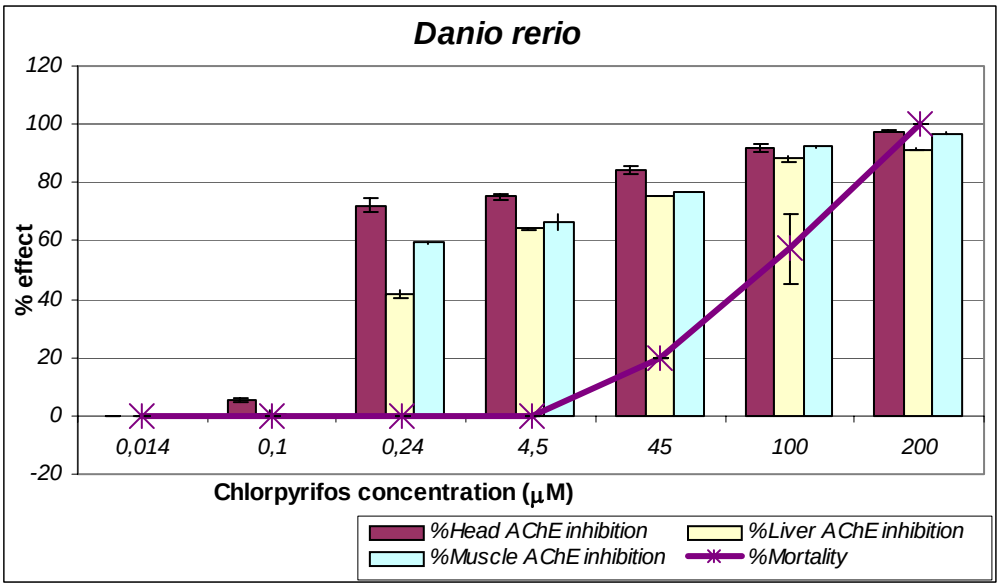
%AChE INHIBITION						
Pesticide	***Head***		***Muscle***		***Liver***	
	IC80 (μM)	**95% confidence limits**	**IC80 (μM)**	**95% confidence limits**	**IC80 (μM)**	**95% confidence limits**
Chlorpyrifos	10,14	(3,11-63,55)	18,47	(5,9-105,76)	34,31	(14,42-113,65)
Carbofuran	90,62	(72,46-126,84)	87,01	(59,88-275,69)	146,82	(75,32-1338,04)
Mixture	63,24	(41,78-132,73)	>45		86,36	(53,19-239,58)

We observed that the acetylcholinesterase inhibition in head, liver and muscle homogenates of Danio rerio is higher than percentage of effect (in that case mortality was considered) after an exposure of 96 h to chlorpyrifos, but for carbofuran and their mixture, AChE inhibition increases at the same rate of percentage of mortality. Values are shown in Figure 58 a-c.

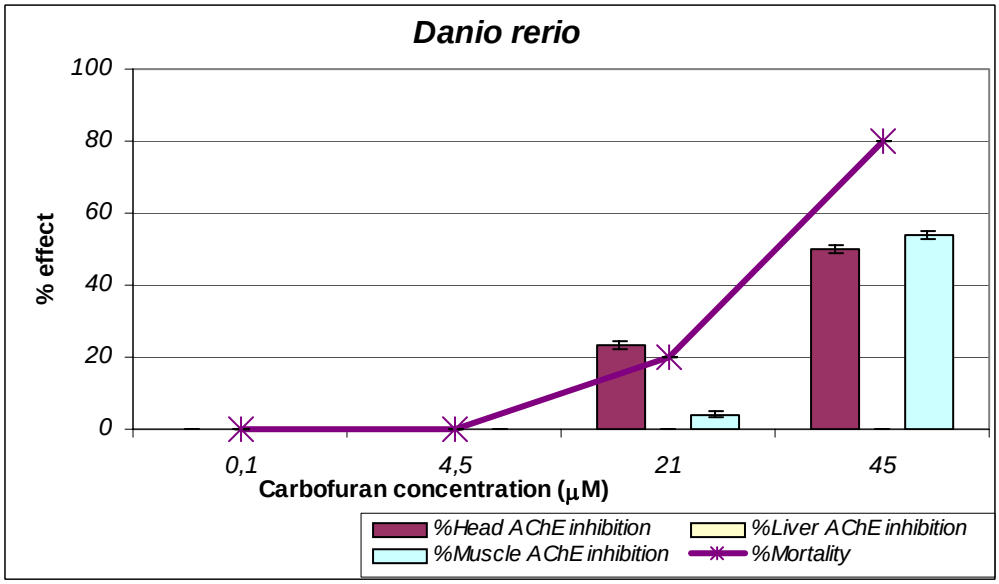
With regard to chlorpyrifos at lower concentrations (0.24 to 4.5 μM) while mortality is zero the AChE inhibition is very important. At 0.24 μM of chlorpyrifos the AChE inhibition is 72.2 % in head, 59.3 % in muscle and 41.7 % in liver. At 4.5 μM the AChE inhibition is 75.1% in head, 66.3% in muscle and 64.1% in liver (Table 3 of the annex 3 of results). This shows a sublethal effect (Figure 58-a). At the maximum concentration tested (200 μM) the mortality is complet and the AChE inhibition is between 91.45 and 97.2%. The AChE inhibition caused by chlorpyrifos is higher than mortality IC50=0.81 μM in head, 1.53 μM in muscle and 2.71 in liver versus LC50= 72.71 μM (Table 14).

On the other hand, for carbofuran and the mixture, it seems that AChE inhibition occurs only when mortality is observed (Table 14 and and Figure 58-b-c). At lower concentrations such as 4.5 μM of carbofuran, there was no mortality (0%) and AChE inhibition in the three tissues is not observed. At 4.5 μM of the mixture there was no mortality as well, and AChE inhibition is 14.5% in head, 0% in muscle and 9.2% in liver. At maximum concentration of carbofuran tested, 45 μM, there is a mortality of 80%, but AChE inhibition is only 49.4 % in head, 53.9 in muscle and 0% in liver. Respect to the highest concentration of the mixture, 45 μM, the mortality is 90% and AChE inhibition is 83.3 % in head, 0% in muscle and 74.1 % in liver (Table 3 of the annex 3 of the results).

a)


Danio rerio
120
100
80
60
40
20
0
-20
% effect
0,014
0,1
0,24
4,5
45
100
200
Chlorpyrifos concentration (µM)
%Head AChE inhibition
%Liver AChE inhibition
%Muscle AChE inhibition
%Mortality

b)


Danio rerio
100
80
60
40
20
0
% effect
0,1
4,5
21
45
Carbofuran concentration (µM)
%Head AChE inhibition
%Liver AChE inhibition
%Muscle AChE inhibition
%Mortality

c)

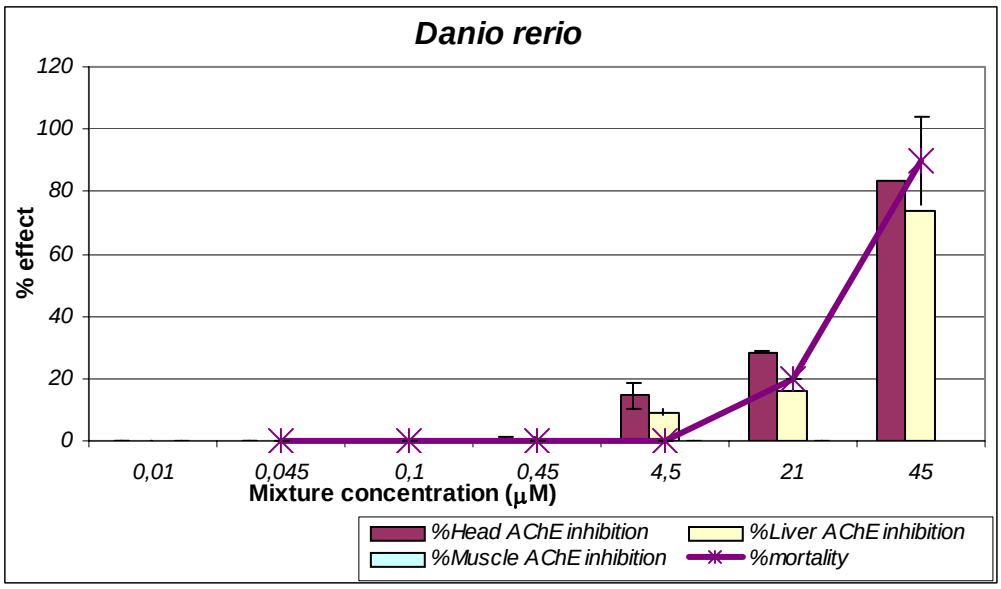


Figure 58. Acetylcholinesterase inhibition of head, liver and muscle homogenates of Danio rerio versus cytotoxicity after an exposure time of 96 h to chlorpyrifos (a), carbofuran (b) and their mixture (c). Values are the mean of two independent experiments, with 2 enzymatic determinations for AChE inhibition and 4 for proteins and their SD represented as standard error bars.

4.4. Acute toxicity in microalgae

The obtained values of percentage growth inhibition for every tested concentration for Chlorella vulgaris, Scenedesmus subspicatus and Selenastrum capricornutum after an exposure period of 72 h to the pesticides chlorpyrifos, carbofuran and mixture of both are presented in Table 4 a, b, c respectively in the annex 4 of results.

The EC20, EC50 and EC80 and their 95% confidence limits obtained from logit method are represented in Table 11 a-c. The graphics of the linear regression obtained from growth inhibition values in Chlorella vulgaris (a), Selenastrum capricornutum (b) and Scenedesmus subspicatus (c) for chlorpyrifos, carbofuran and their mixture were represented in Figures 59, 60 and 61.

We can observe that carbofuran is more toxic than chlorpyrifos in the three tested species (there are statistical differences using ANOVA $p<0.05$). In Chlorella vulgaris, the concentration that causes an inhibition of 20% in growth is 48.51 μM for chlorpyrifos, 9.63 μM for carbofuran and 4.81 μM for the mixture. The concentration that causes the 50% of mortality is 127.5 μM for chlorpyrifos, 27.04 μM for carbofuran and 24.94 μM for the mixture. The concentration that causes the 80% of mortality is 335.16 μM for chlorpyrifos, 75.89 μM for carbofuran and 129.42 μM for the mixture.

In Selenastrum capricornutum, the concentration that causes an inhibition of 20% in growth is 44.24 μM for chlorpyrifos, 11.22 μM for carbofuran and 13.12 μM for the mixture. The concentration that causes the 50% of mortality is 132.34 μM for chlorpyrifos, 62.93 μM for carbofuran and 46.22 μM for the mixture. The concentration that causes the 80% of mortality is 395.86 μM for chlorpyrifos, 352.96 μM for carbofuran and 162.81 μM for the mixture.

In Scenedesmus subspicatus, the concentration that causes an inhibition of 20% in growth is 32.62 μM for chlorpyrifos, 15.83 μM for carbofuran and 14.28 μM for the mixture. The concentration that causes the 50% of mortality is 71.21 μM for chlorpyrifos, 56.81 μM for carbofuran and 51.33 μM for the mixture. The concentration that causes the 80% of mortality is 155.49 μM for chlorpyrifos, 203.83 μM for carbofuran and 184.44 μM for the mixture.

Table 11. Chlorella vulgaris, Scenedesmus subspicatus, Selenastrum capricornutum. Relation of EC 20, 50 and 80 at 72 h (expressed in μM) and their 95% confidence limits.

a)

Pesticide	*Chlorella vulgaris*		*Selenastrum capricornutum*		*Scenedesmus subspicatus*	
	EC20 (μM)	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**	**EC20 (μM)**	**95% confidence limits**
Chlorpyrifos	48,51	(38,37-57,77)	44,24	(39,60-48,70)	32,62	(28,12-36,84)
Carbofuran	9,63	(8,18-11,09)	11,22	(9,57-12,91)	15,83	(14,11-17,56)
Mixture	4,81	(3,92-5,71)	13,12	(10,79-15,40)	14,28	(12,74-15,81)

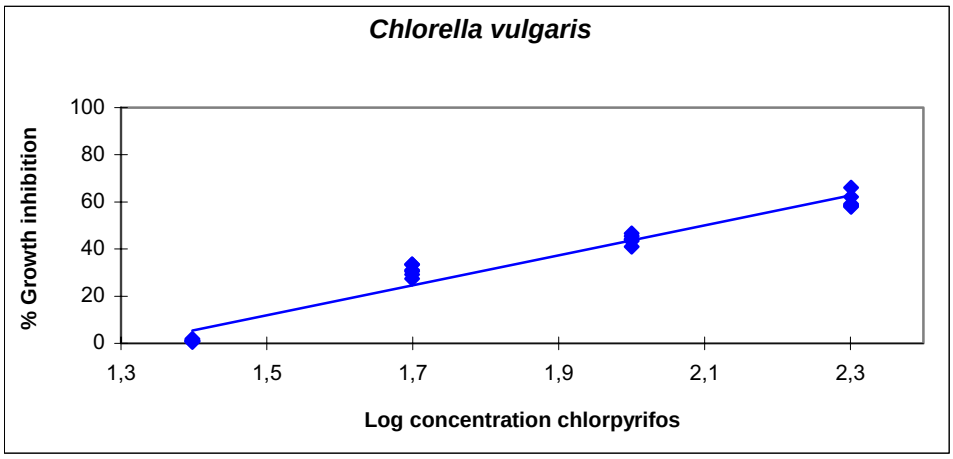
b)

Pesticide	*Chlorella vulgaris*		*Selenastrum capricornutum*		*Scenedesmus subspicatus*	
	EC50 (μM)	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**	**EC50 (μM)**	**95% confidence limits**
Chlorpyrifos	127,50	(109,60-153,19)	132,34	(122,10-144,71)	71,21	(65,24-77,74)
Carbofuran	27,04	(24,46-29,79)	62,93	(57,33-69,32)	56,81	(52,82-61,15)
Mixture	24,94	(22,74-27,36)	46,22	(40,70- 53,26)	51,33	(47,23-56,16)

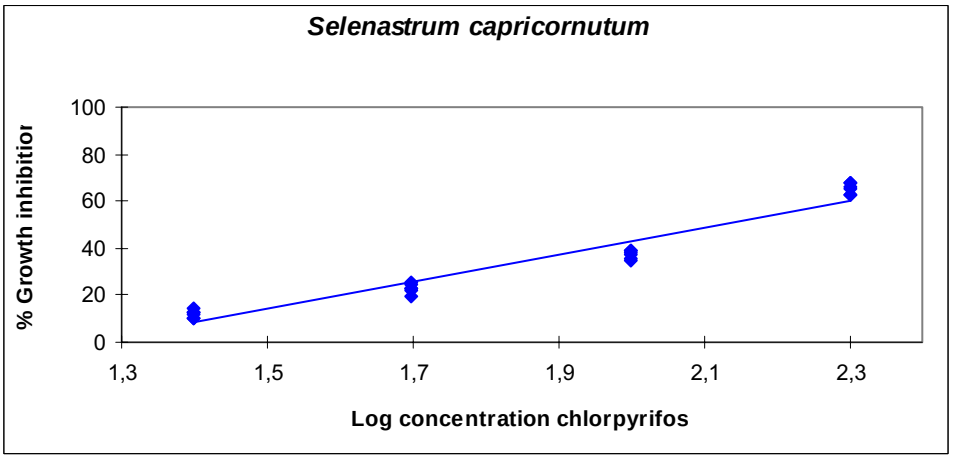
c)

Pesticide	*Chlorella vulgaris*		*Selenastrum capricornutum*		*Scenedesmus subspicatus*	
	EC80 (μM)	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**	**EC80 (μM)**	**95% confidence limits**
Chlorpyrifos	335,16	(257,07-494,63)	395,86	(338,14-478,86)	155,49	(137,61-180,49)
Carbofuran	75,89	(67.42-86.72)	352,96	(296,78-430,88)	203,83	(181,81-231,60)
Mixture	129,42	(108,97-158,74)	162,81	(129,35-218,60)	184,44	(157,6-221,69)

a)



b)



c)

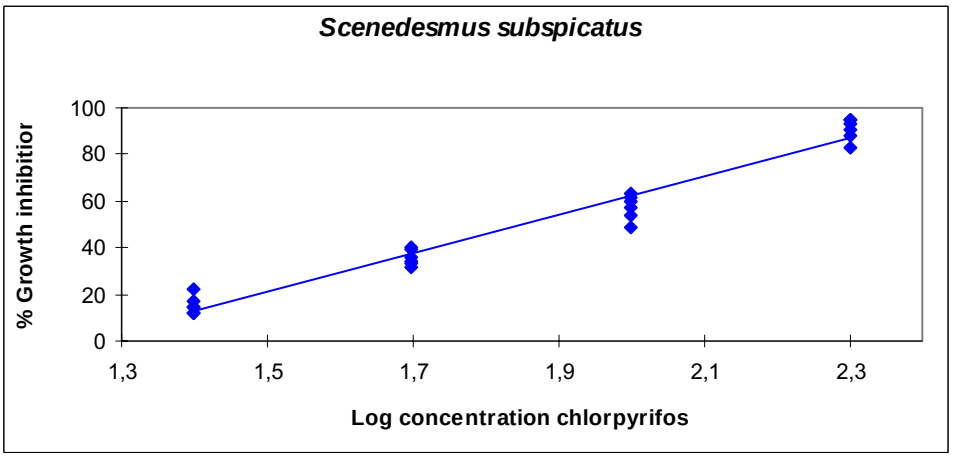
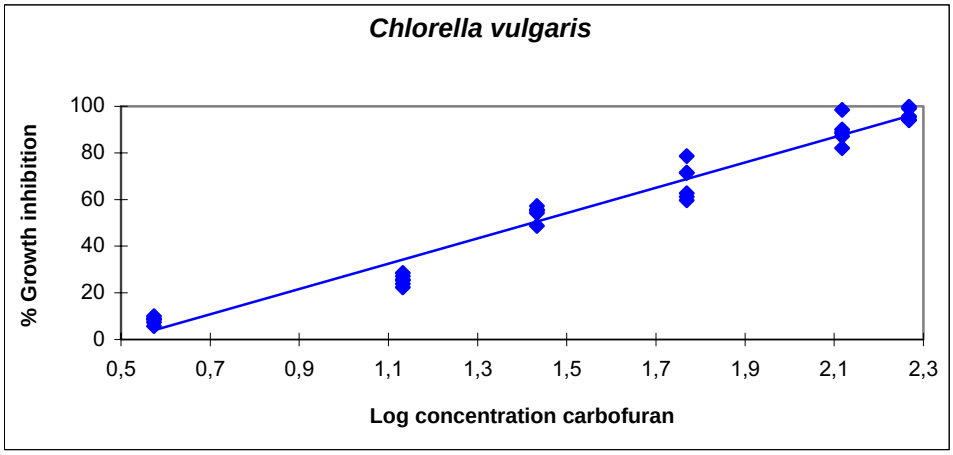
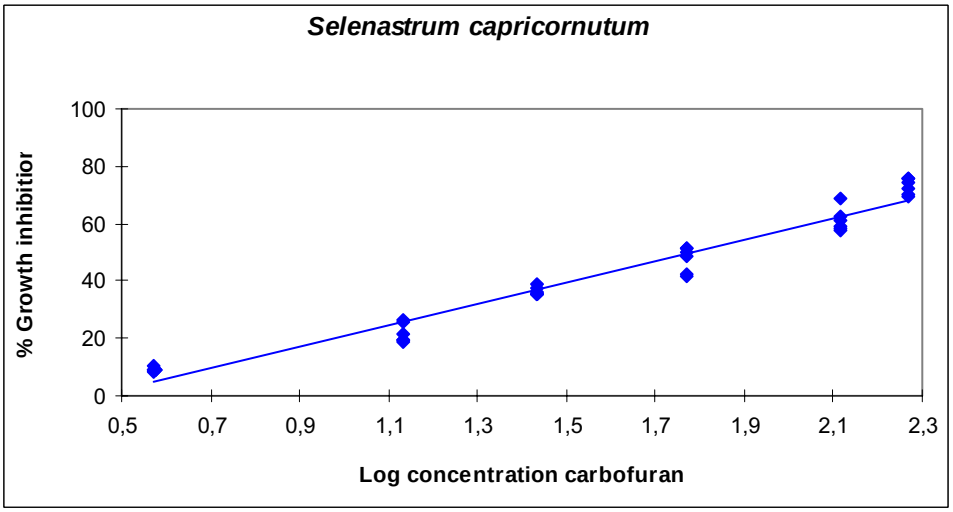


Figure 59: Graphic representation of the linear regression obtained from the chlorpyrifos growth inhibition values in Chlorella vulgaris (a), Selenastrum capricornutum (b) and Scenedesmus subspicatus(c). Percentage of growth inhibition is represented versus logarithm of chlorpyrifos concentrations.

a)



b)



c)

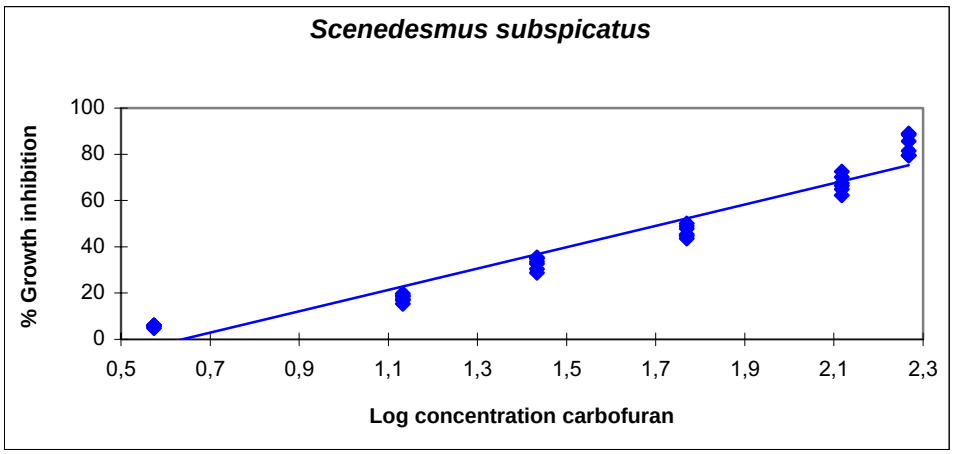
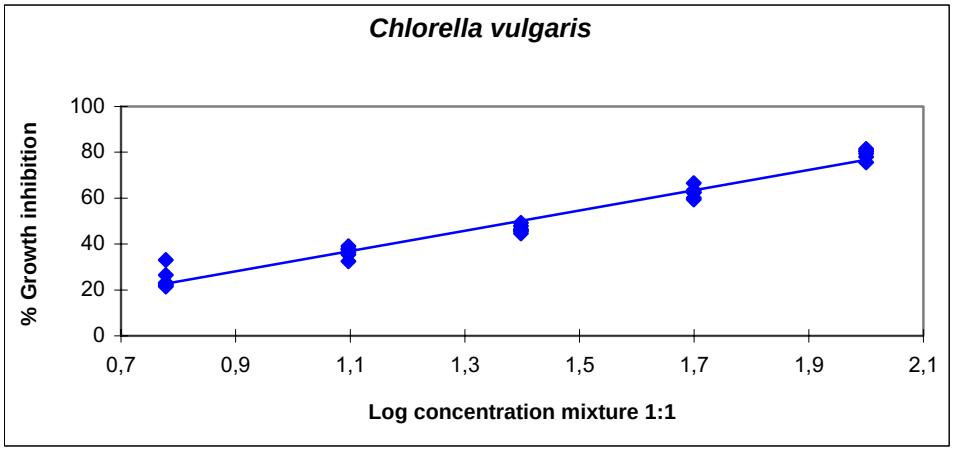
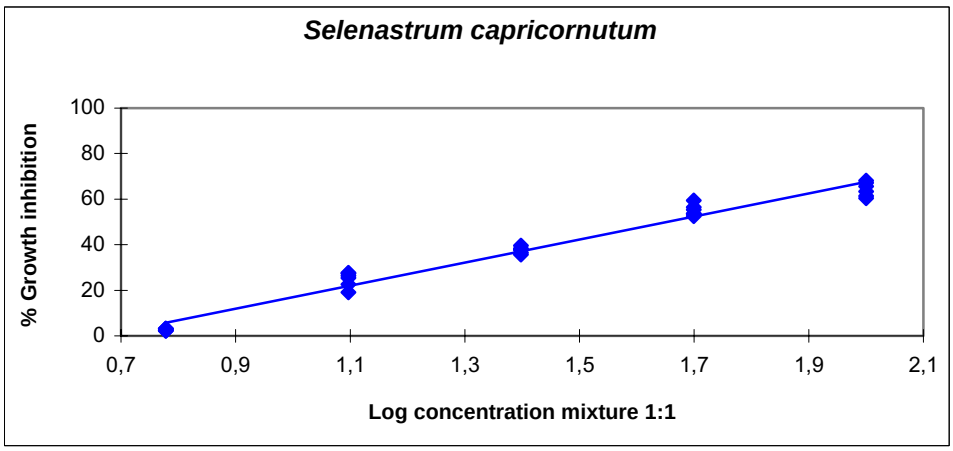


Figure 60: Graphic representation of the linear regression obtained from the carbofuran growth inhibition values in Chlorella vulgaris (a), Selenastrum capricornutum (b) and Scenedesmus subspicatus(c). Percentage of growth inhibition is represented versus logarithm of carbofuran concentrations.

a)



b)



c)

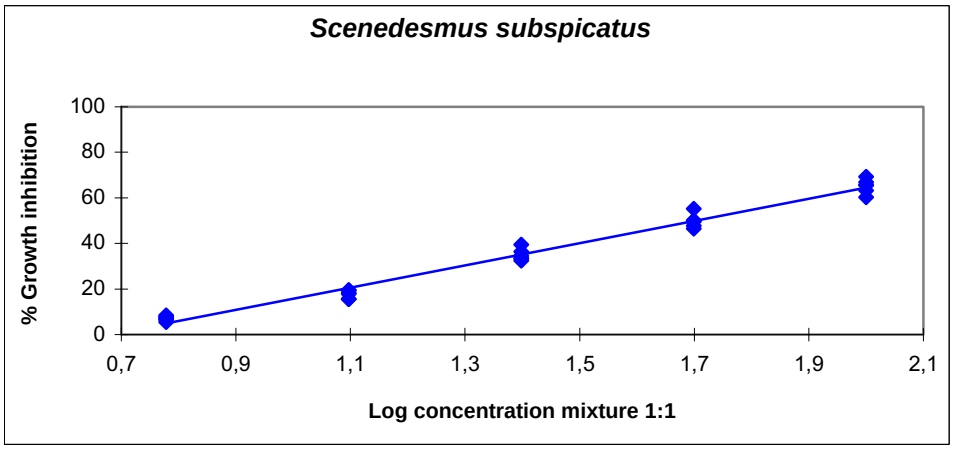


Figure 61: Graphic representation of the linear regression obtained from the mixture growth inhibition values in Chlorella vulgaris (a), Selenastrum capricornutum (b) and Scenedesmus subspicatus (c). Percentage of growth inhibition is represented versus logarithm of the mixture chlorpyrifos-carbofuran concentrations.

4.5. Acute toxicity in bacteria. *Vibrio fischeri*

The obtained results of acute toxicity for Vibrio fischeri after an incubation period of 5 and 15 min to the pesticides chlorpyrifos, carbofuran and their mixture are showed in Table 5 in the annex 5 of results. The toxicity values are represented by the gamma value (Γ), which is the ratio of the light lost at time t to the light remaining at time t for a given sample concentration.

A double log plot of Γ versus concentration was used to calculate EC50 values, i.e. the concentration of toxicant where Γ =1. (Figure 62 a-c)

The results of the acute toxicity tests are reported as the EC50 values (μM) at 5 and 15 min exposure (Table 12). Each value was determined a minimum of three times with individually prepared test solutions, usually on three or more different days, and the mean of the values is given.

Table 12. Vibrio fischeri. Relation of obtained EC50 and their 95% confidence limits at 5 and 15 minuts (μM) of the pesticides chlorpyrifos, carbofuran and the mixture.

Pesticide	EC*50* (μM)"		EC*50* (μM)"	
	5 min	95% confidence limits	15 min	95% confidence limits
Chlorpyrifos	*212,65*	*(201,16-225,10)*	*195,79*	*(85,92-206,31)*
Carbofuran	*63,58*	*(56,31-72,21)*	*56,10*	*(49,81-63,34)*
Mixture	*70,57*	*(63,70-77,71)*	*62,32*	*(52,72-73,05)*

Pesticide toxicities determined with the Microtox™ test are noted in Table 13 as EC20 and EC80 values for 5 and 15 min incubation. Generally the ECxx values for 5 min incubation are greater than for 15 min incubation (Table 12 and 13).

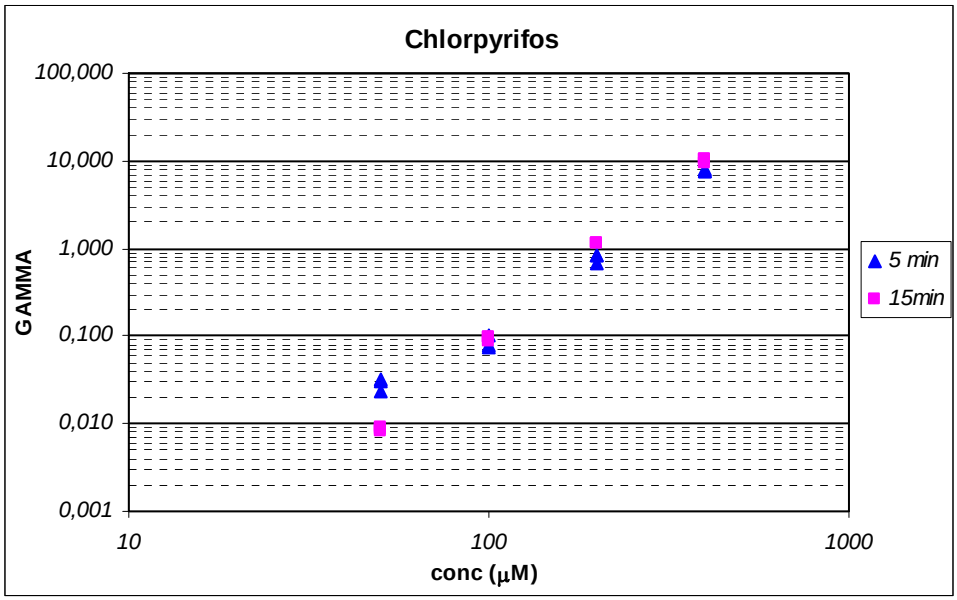
On the other hand, carbofuran is more toxic than chlorpyrifos. At 5 min the EC50 is 63.58 μM for carbofuran, 70.57 μM for the mixture and 212.65 μM for chlorpyrifos.

Table 13. Vibrio fischeri. Relation of obtained EC20 and EC80 at 5 and 15 minuts (μM) of the pesticides chlorpyrifos, carbofuran and the mixture.

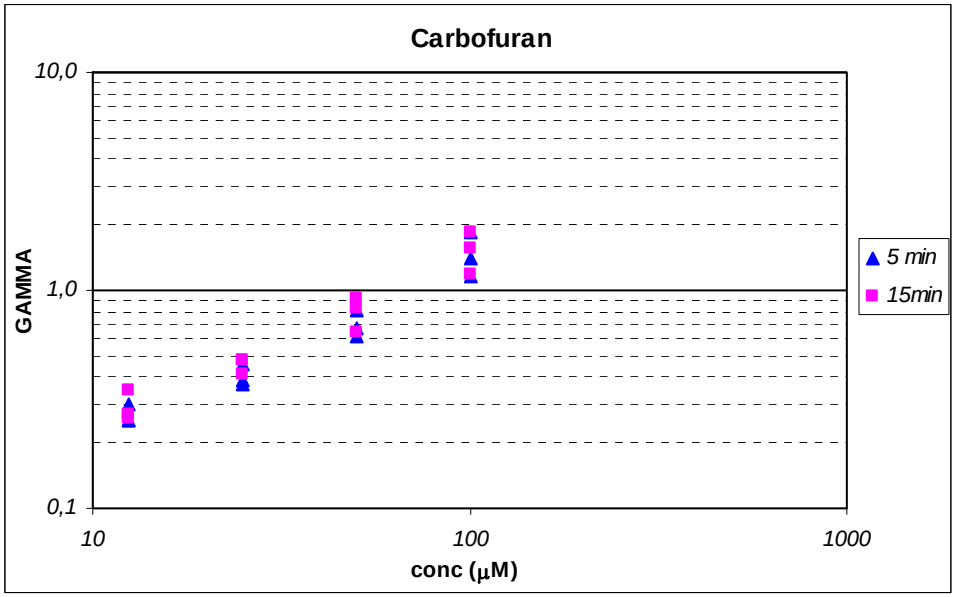
Pesticide	EC20 (μM)		EC80 (μM)	
	Incubation time (min)		Incubation time (min)	
	5	15	5	15
Chlorpyrifos	*102,46*	*102,34*	*337,09*	*294,82*
Carbofuran	*5,78*	*5,37*	*288,81*	*246,60*
Mixture	*21,73*	*19,96*	*229,21*	*194,53*

The Figures 62 a-c represents the logaritm of gamma versus logartim of the concentrations of pesticide.

a)



b)



c)

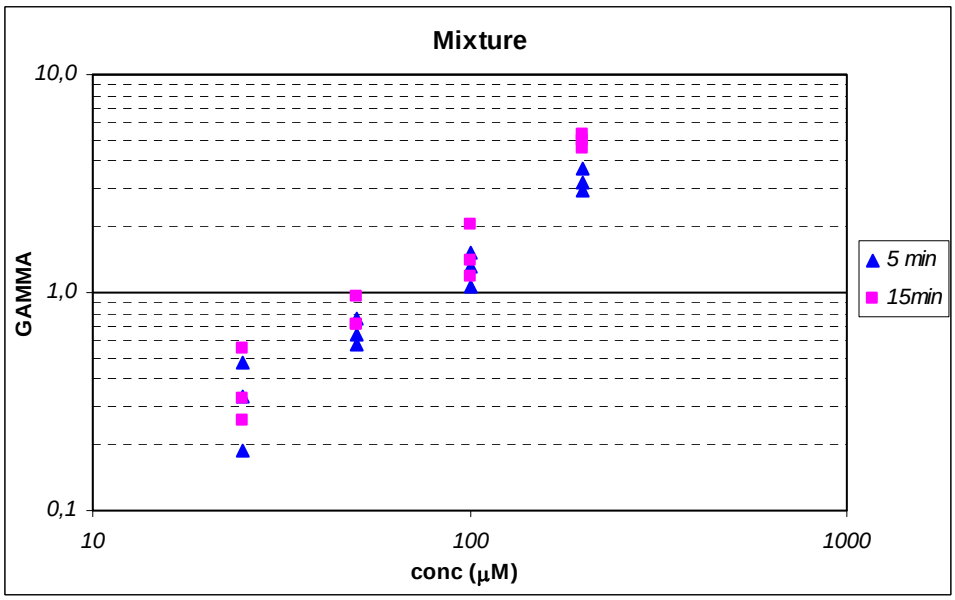


Figure. 62. Representative double log plots of values vs. concentration for the Microtox™ assay: (a) chlorpyrifos (5–15 min incubation); (b) carbofuran (5–15 min incubation); (c) mixture 1:1 (5-15 min incubation)

4.6. Resum results

The obtained toxicity values for all the tested species after an exposure to chlorpyrifos, carbofuran and their mixture is shown in Table 14 and represented in Figures 63 and 64.

Table 14. Toxicity values for all the tested species after an exposure to chlorpyrifos, carbofuran and their mixture. NR= Neutral red uptake, KB=Kenacid blue, ATP=Adenosin triposphate, CI= Cytotoxicity index, IC=Inhibition of AChE

CHLORPYRIFOS					
In vivo					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Bacteria	*Vibrio fischeri*		*5 min EC50*	212,65	(201,16-225,10)
			15 min EC50	195,79	(85,92-206,31)
Algae	*Chlorella vulgaris*		*72h EC50*	127,50	(109,60-153,19)
	Selenastrum capricornutum		*72h EC50*	132,34	(122,10-144,71)
	Scenedesmus subspicatus		*72h EC50*	71,21	(65,24-77,74)
Fish	*Danio rerio*		*96 h LC50*	72,71	(57,83-87,99)
	Danio rerio	*Head*	*96 h IC50*	0,81	(0,20-2,59)
		Liver	*96 h IC50*	2,71	(1,16- 6,03)
		Muscle	*96 h IC50*	1,53	(0,45-4,64)
In vitro					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Primary bovine cells	*Bos taurus (granulosa cells)*	*NR*	*48h EC50*	16,16	(9,53-27,86)
		KB	*48h EC50*	26,87	(15,28-53,56)
		ATP	*48h EC50*	22,27	(14,30-36,31)
		CI	*48h EC50*	36,2	(24,08-61,55)
		IC	*48h IC50*	20,96	(9,20-58,63)
Fish cell line	*RTG-2 Oncorhynchus mykiss*	*NR*	*48h EC50*	17,62	(12,93-24,95)
		KB	*48h EC50*	30,13	(21,51-44,43)
		ATP	*48h EC50*	19,49	(14,42-27,36)
		CI	*48h EC50*	35,94	(25,05-54,83)
		IC	*48h IC50*	1,044	(0,61-1,76)

CARBOFURAN					
In vivo					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Bacteria	*Vibrio fischeri*		*5 min EC50*	63,58	(56,31-72,21)
			15 min EC50	56,10	(49,81-63,34)
Algae	*Chlorella vulgaris*		*72h EC50*	27,04	(24,46-29,79)
	Selenastrum capricornutum		*72h EC50*	62,93	(57,33-69,32)
	Scenedesmus subspicatus		*72h EC50*	56,81	(52,82-61,15)
Fish	*Danio rerio*		*96 h LC50*	28,88	(24,35-33,54)
	Danio rerio	*Head*	*96 h IC50*	43,23	(38,2-50,77)
		Liver	*96 h IC50*	57,34	(38,18-167,73)
		Muscle	*96 h IC50*	47,65	(38,15-78,77)
In vitro					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Primary bovine cells	*Bos taurus (granulosa cells)*	*NR*	*48h EC50*	17,37	(10,81-31,5)
		KB	*48h EC50*	13,53	(8,69-23,14)
		ATP	*48h EC50*	15,90	(10,6-26,3)
		CI	*48h EC50*	23,05	(14,03-45,04)
		IC	*48h IC50*	53,78	(33,51-96,86)
Fish cell line	*RTG-2 Oncorhynchus mykiss*	*NR*	*48h EC50*	9,80	(5,36-19,89)
		KB	*48h EC50*	17,38	(9,16-39,48)
		ATP	*48h EC50*	10,94	(5,75-24,0)
		CI	*48h EC50*	19,36	(11,37-37,34)
		IC	*48h IC50*	31,44	(17,65-67,44)

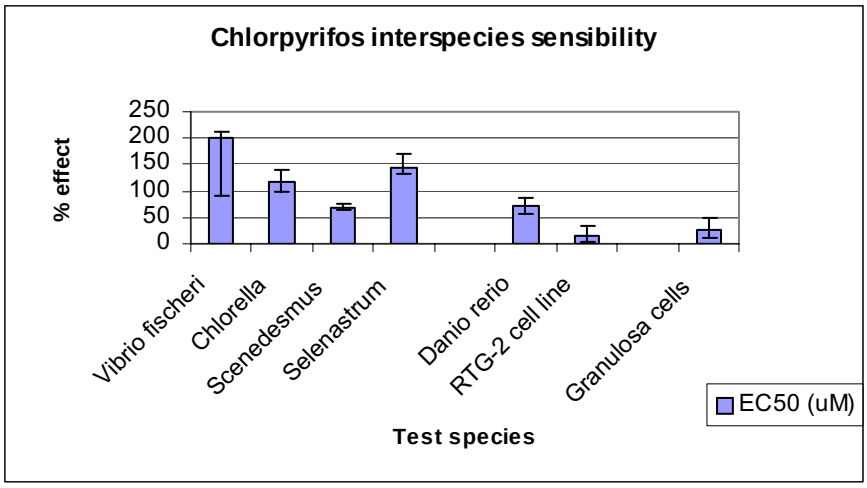
MIXTURE					
In vivo					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Bacteria	*Vibrio fischeri*		*5 min EC50*	70,57	(63,70-77,71)
			15 min EC50	62,32	(52,72-73,05)
Algae	*Chlorella vulgaris*		*72h EC50*	24,94	(22,74-27,36)
	Selenastrum capricornutum		*72h EC50*	46,22	(40,70- 53,26)
	Scenedesmus subspicatus		*72h EC50*	51,33	(47,23-56,16)
Fish	*Danio rerio*		*96 h LC50*	32,45	(23,29-52,48)
	Danio rerio	*Head*	*96 h IC50*	22,72	(16,59-32,70)
		Liver	*96 h IC50*	32,45	(23,30-52,48)
		Muscle	*96 h IC50*	>45	
In vitro					
Organism	**Test species**		**Exposure time and endpoint**	**Toxixity value (μM)**	**95% confidence limits**
Primary bovine cells	*Bos taurus (granulosa cells)*	*NR*	*48h*	16,24	(11,63-23,98)
		KB	*48h*	24,57	(16,75-39,10)
		ATP	*48h*	14,64	(10,47-21,62)
		CI	*48h*	21,23	(15,07-32,21)
		IC	*48h*	54,47	(34,97-95,61)
Fish cell line	*RTG-2 Oncorhynchus mykiss*	*NR*	*48h*	11,69	(7,37-19,70)
		KB	*48h*	20,52	(16,06-26,89)
		ATP	*48h*	9,81	(6,5-15,51)
		CI	*48h*	21,23	(16,3-28,45)
		IC	*48h*	32,42	(22,31-50,63)

Furthermore, the toxicologic interactions between chlorpyrifos and carbofuran in all the tested organisms and used endpoints are shown in tabe 15. The obtained toxicity units (TU) for each pesticide single and in mixture are showed in Table 7 of the annex 6 of results.

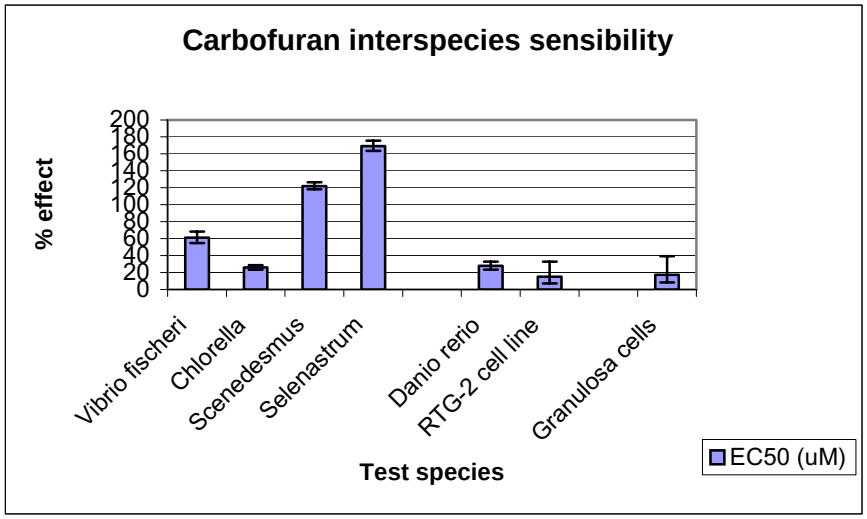
Table 15. Interactions of toxicity response to mixture chlorpyrifos-carbofuran in all the organisms tested. Yhe values of toxicity units are shown in table x of the annex of results.

Interactions of toxicity response to mixture chlorpyrifos-carbofuran				
In vivo				
Organism	**Test species**		**Exposure time and endpoint**	**Toxicology interaction**
Bacteria	*Vibrio fischeri*		*5 min EC50*	*Synergistic*
			15 min EC50	*Synergistic*
Algae	*Chlorella vulgaris*		*72h EC50*	*Synergistic*
	Selenastrum capricornutum		*72h EC50*	*Synergistic*
	Scenedesmus subspicatus		*72h EC50*	*Synergistic*
Fish	*Danio rerio*		*96 h LC50*	*Synergistic*
	Danio rerio	*Head*	*96h IC50*	*Antagonistic*
		Liver	*96h IC50*	*Antagonistic*
		Muscle	*96h IC50*	*Antagonistic*
In vitro				
Organism	**Test species**		**Exposure time and endpoint**	**Toxicology interaction**
Primary bovine cells	*Bos taurus (granulosa cells)*	*NR*	*48h EC50*	*Synergistic*
		KB	*48h EC50*	*Synergistic*
		ATP	*48h EC50*	*Synergistic*
		CI	*48h EC50*	*Synergistic*
		IC	*48h IC50*	*Antagonistic*
Fish cell line	*RTG-2 Oncorhynchus mykiss*	*NR*	*48h EC50*	*Synergistic*
		KB	*48h EC50*	*Synergistic*
		ATP	*48h EC50*	*Synergistic*
		CI	*48h EC50*	*Synergistic*
		IC	*48h IC50*	*Antagonistic*

a)



b)



c)

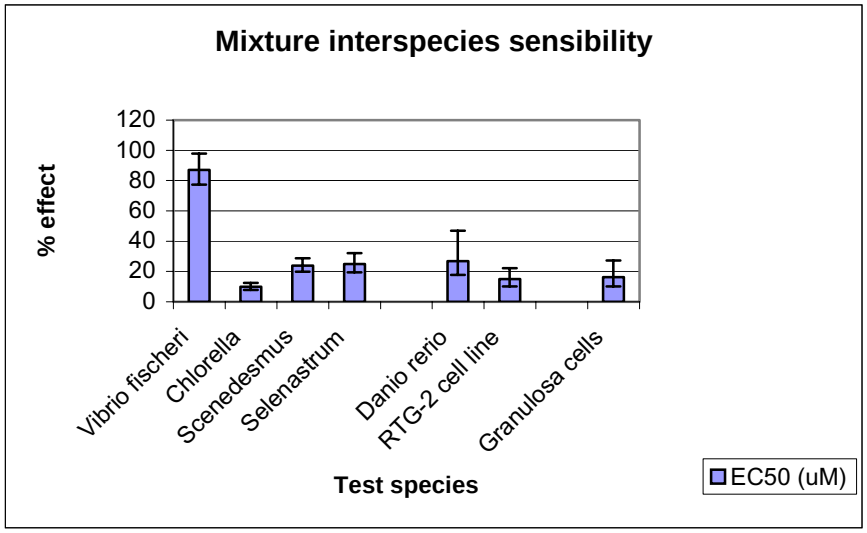
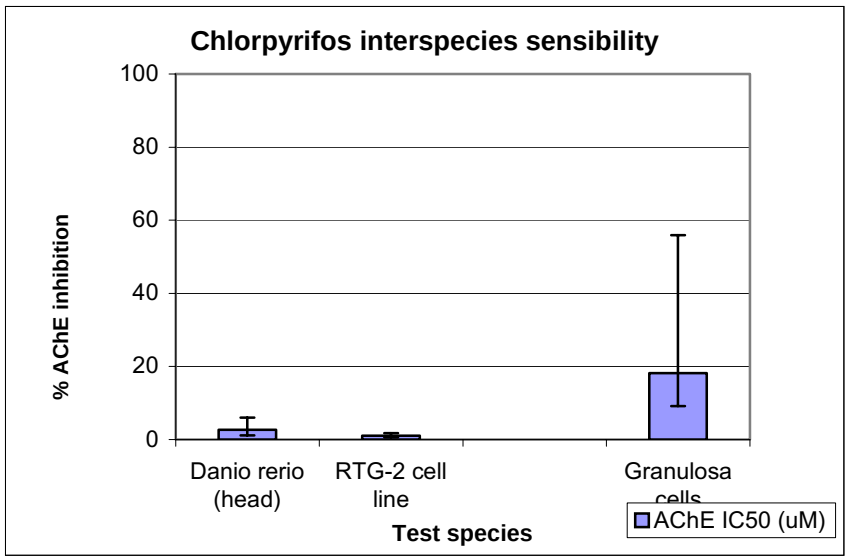
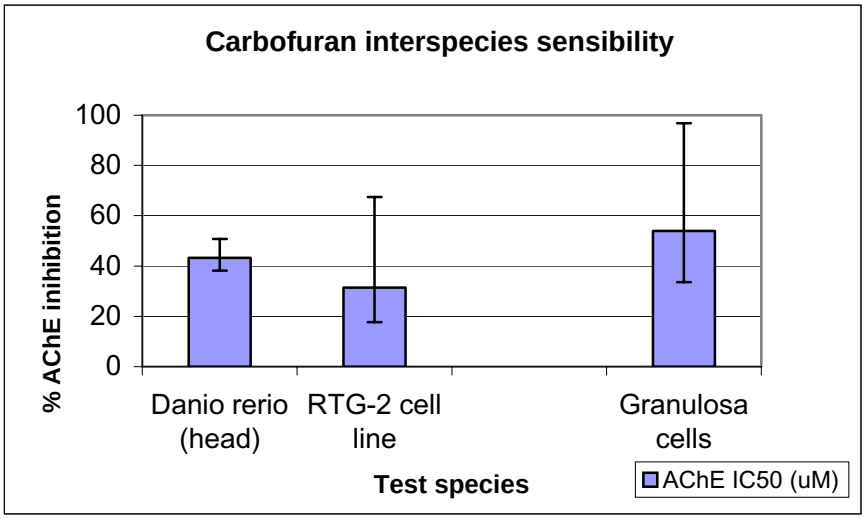


Figure 63. Obtained EC50 values and their confidence intervals in bars in all the tested species showng different sensibility to chlorpyrifos (a), carbofuran (b) and their mixture (c).

a)



b)



c)

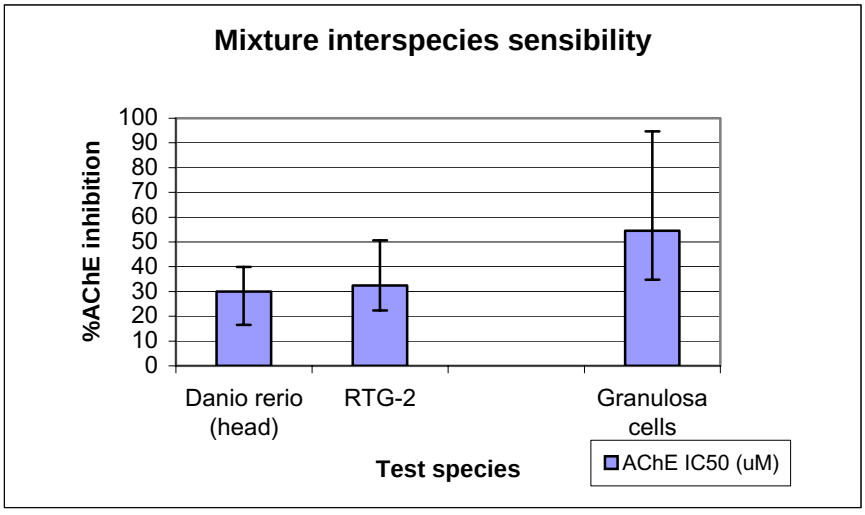


Figure 64. Obtained AChE IC50 values and their confidence intervals in bars in all the tested species showing different sensibility to chlorpyrifos (a), carbofuran (b) and their mixture (c).

Discussion

5. Discussion.

5.1. IN VITRO ASSAYS

Inherent economic and ethical constraints associated with tests on live animals such as the in vivo fish lethality assay, has encouraged the continued development of acute in vitro bioassays. Consequently, the utilisation of in vitro test systems such as primary cultures and immortal cell lines are of growing importance in aquatic ecotoxicology.

Interactions between chemical contaminants and biological systems initially take place at the cellular level. The use of in vitro cell cultures for ecotoxicological assessment can therefore be a valuable tool for the early and sensitive detection of chemical exposure (Segner and Braunbeck, 1998).

Besides primary cell cultures derived from various tissues, fish cell lines are becoming the most important in vitro tools in aquatic ecotoxicology (Fent, 2001).

Cell line-based cytotoxicity assays are important in contributing to the evaluation of the environmental hazard of chemicals and environmental samples in combination with other ecotoxicological test systems. For this reason, in this study we employed a fish cell line and a primary cell culture using various endpoints to evaluate the potential cytotoxic effect of different pesticides at the cellular level and a battery of organisms from different trophic levels, such as algae (Chlorella vulgaris, Selenastrum capricornutum and Scenedesmus subspicatus), bacteria (Vibrio fischeri) and vertebrates, zebra fish (Danio rerio).

Fish cell lines may be useful for prediction of acute fish toxicity. In general, fish cytotoxicity tests are very suitable for the screening of chemicals, at least for an initial hazard assessment, because of the simple and rapid measurements of a large sample number in 96-well plates. In several studies cytotoxicity was found to be correlated with in vivo acute fish toxicity in different cell lines (Babich and Borenfreund, 1987; Dierickx and Van den Vyver, 1991; Brandao et al., 1992; Segner and Lenz, 1993; Brüschweiler et al., 1995; Castaño et al., 1996; Fent and Hunn, 1996).

RTG-2 from rainbow trout (Oncorhynchus mykiss) has been used in many studies (Alañón et al, 2005; Riva et al., 2005; Babín et al., 2005; Sánchez-Fortún et al., 2005;

Shúilleaháin et al, 2004; Alañón et al., 2003, 2001; Fent, 2001; Araujo et al., 2000; Lee, et al., 1998; López et al, 1998; Vega et al., 1995; Castaño et al., 1994a; 1994b ; 1995; 1996).

In this work we were interested in using a gonadal cell line like RTG-2, which is obtained from a mix of testis and ovary. In that way we can compare better with the response of granulosa primary cells obtained from bovine ovaries. We couldn't use an established cell line from zebra fish because there are only five established cell lines available: SJD.1, AB.9 from caudal fin; ZEMS and ZF4 from embryo and ZFL from liver and neither of them are derived from gonads.
On the other hand, bovine granulosa cells have been used in a lot of studies (Stacchezzini et al, 2006; Alañón et al, 2005; Vanholder et al, 2005; Tiemann et al., 1996; van Wezel et al., 1996, Langhout et al, 1991; Luck et al, 1990).
Bovine ovaries can be collected in a nearby slaughterhouse. Granulosa cells are easily extracted from the ovaries and are viable for some weeks maintaining their differentiated functions.

We studied if there were significant differences respect to the continuous fish cell line RTG-2. Both types of cells are from gonadal origin, but ones are obtained from fish and the others from mammalians. We considered that it could be interesting in order to compare the two types of cells because if unique toxic mechanism occurs in different species, this information can be used to assess the impact of toxicants on both the species and the ecosystem. Identifying unique toxic mechanisms or susceptibilities for species can be of ecotoxicological value.

Cell viability was evaluated using traditional cytotoxicity assays like the highest test pesticide concentration (HTD) that causes the minimum morphological changes to cells (Shúilleabháin et al, 2004; Liebsch and Spielmann, 1995), the neutral red stain uptake (NRU) (Borenfreund and Puerner, 1985; Maracine and Segner, 1998), the cell detachment using the Kenacid blue protein assay (KBP) (Knox et al., 1986) and the ATP determination (Castaño et al., 1994a).

Genotoxicity was assessed using the single-cell gel electrophoresis or Comet assay. It is a genotoxic indicator method since it allows the rapid, relatively simple, and sensitive measurement of DNA damage in eukaryotic cells (McKelvey et al., 1993; Fairbairn et al., 1995; Tice et al., 2000).

The enzyme acetylcholinesterase was characterised and its inhibition was measured as biomarker of the sublethal effects produced by the neurotoxic pesticides of this study (Barata, 2001, 2004).
The first parameter measured in both types of cells was the HTD, which is defined as the highest test pesticide concentration that causes the minimum morphological changes to cells (Shúilleabháin et al, 2004; Liebsch and Spielmann, 1995). The light microscopic appearance of RTG-2 cell line and bovine granulosa cells following 48 h exposure to the pesticides are shown in Figures 14, 15, and 16 for RTG-2 and 30, 31, 32 for bovine granulosa cells. The respective HTD values are also reported in Table 1 for RTG-2 and table 5 for bovine granulosa cells.

A discernible morphological difference was apparent between control cells and those exposed to 1 μM (RTG-2) and 2.2 μM (Granulosa cells) of chlorpyrifos, 0.45 μM of carbofuran for both cells and 0.45 μM for the mixture chlorpyrifos-carbofuran also for both types of cells.

These differences were observed as a subtle change in the cell density of the monolayer when compared to control as opposed to obvious cell damage seen in the Figure 16 and 32 (maximum effect).

Despite the subjectivity involved with identifying morphological changes, light microscopic inspection is undoubtedly a useful adjunct in verifying the results of the following colorometric tests (Hollert et al., 2000; Bieberstein and Braunbeck, 1998).

The incorporation of the Neutral Red dye by the lysosomes of living cells and the staining of the total amount of cellular proteins were shown in Figure 17, 18 and 33, 34 for RTG-2 and bovine granulosa cells, respectively.

Figures 19 a-c and Figures 35 a-c show correlation among the endpoints neutral red, kenacid blue and ATP determination for RTG-2 and bovine granulosa cells, respectively. In general, we can observe a tendency being the neutral red uptake and the ATP content the most sensitive endpoints and protein determination the less sensitive (no statistically significant differences using one-way ANOVA ($p < 0.05$)). Cytotoxicity is expected to produce a decrease in cell viability (measured by neutral red) followed by cell detachment (measured by total protein assays). Therefore, good agreement between protein and neutral red effect is generally expected. However,

detachment requires some time, and in short-term tests, non-viable cells can still be attached to the plate and diminish the capability of protein measurement to identify toxic effects. Under some conditions, e.g. by exposure to some solvents, death cells can be fixed on the plastic surface and are accounted as live cells in the protein measurements. Although both mechanisms could explain the lack of protein response at doses severely affecting neutral red, the first hypothesis seems to be more likely.
This observation is according with the published results of previous studies (Castaño et al., 1996; Sauvant et al., 1997; López et al., 1998; Gibert et al., 2001; Alañón et al., 2001; Fent, 2001; Shúilleabháin et al., 2004; Riva el al., 2005; Babín et al., 2005).

Table 2 and 6 show the obtained EC20, EC50 and EC80 values and its 95% confidence intervals using these endpoints and the obtained Castaño's cytotoxicity index for RTG-2 and bovine granulosa cells, respectively.

In both types of cells, according to the values of the cytotoxicity index (CI) carbofuran (EC50 19.36 μM for RTG-2 and 23.05 for granulosa cells) is more toxic than chlorpyrifos (EC50 35.95 μM for RTG-2 cell line and 36.2 μM for granulosa cells). This concurs with the results of Parran et al., 2005, that found an EC50 value for chlorpyrifos of 10 μM in bovine microvascular endothelial cells using the lactate dehydrogenase (LDH) assay. Babín et al., 2005 reported an EC50 value of 4.5 μM (2.85-7.1 μM) for chlorpyrifos in RTG-2 cell line using the neutral red endpoint.

The sum of individual chlorpyrifos and carbofuran TU's was lower than 1 in both cells (Table 7 of annex 6 of results), suggesting that these pesticides when in mixture induce synergistic effects on cytotoxicity.

Data suggest that in vitro testing using a battery of endpoints can be a cost-effective solution for screening the toxicity of pesticides on fish.

Several authors such as Shúilleabháin et al. 2004, Lopéz et al. 1998, Castaño et al., 1996, Vega et al. 1995 recommend the determination that more than one endpoint in cytotoxicity assays in order to avoid under or overestimation of cytotoxicity.

On the other hand, measurement of acetylcholinesterase (AChE) activity levels is a useful, dose dependent means of monitoring exposure to organophosphorous and carbamate compounds (Carlock et al., 1999). These pesticides exert acute toxicity by

inhibiting the enzyme acetylcholinesterase, a serine hydrolase found in neuromuscular junctions. This leads to susceptible species in accumulation of the neurotransmitter acetylcholine and subsequent hyperpolarisation of the post -synaptic membrane.
Acetylcholinesterases exhibit genetic and molecular polymorphism and their distributions and physiological roles differ among species (Massoullie; Scaps and Forget). As a consequence, the degree of inhibition associated with toxicity is highly variable (Lundebye et al., 1997) depending on the studied specie.

However, the enzyme is not restricted to inactivate acetylcholine and accumulated evidence increasingly points to non-catalytic roles (Soreq and Seidman, 2001).

AChE also exists in WRL-10A cultured fibroblasts a subline of L-929 mouse fibroblasts (Bartos et al., 1976), human erythrocytes (Ott et al., 1982), T lymphocytes (Szelenyi et al., 1982) and murine thrombocytic cells (Paulus et al., 1981), in granulosa cells (Mayerhorfer et al., 2005, 2003; Fritz, 2001; Silver, 1978), also Jin QH, 2004 studied the potential function of acetylcholinesterase in Normal Rat Kidney (NRK) cells. In all these cells, the enzyme has no obvious relations to its classic function.

Mayerhorfer et al., 2003, 2005 have proved that acetylcholine (ACh) is a non-neuronal intraovarian signalling molecule, produced by granulosa cells and which appears to act as signalling factor in the growing follicle. Thus, granulosa cells are an unexpected non-neuronal source of ACh in the ovary.

Before the acetylcholinesterase assay, we performed the acetylcholinesterase activity staining according to the method of Karnovsky and Roots (1961) in order to detect if there were AChE activity in RTG-2 and bovine granulosa cells.
The observed staining in the three different treatments is shown in Table 3 for RTG-2 and Table 7 for granulosa cells.

Treatment 1 consists of the control cells. In this treatment there was only staining solution without substrate acetylthiocholine (AcSCh). Therefore as we expected, the staining was negative and no colour was observed as shows Figure 20-a and Figure 36-a for RTG-2 and granulosa cells, respectively.

In Treatment 2 (stainig solution with substrate acetylthiocholine (AcSCh)) the staining was positive, it means that there was the formation of a brown precipitate in the cells as

shown Figure 20-b and 36-b for RTG-2 and granulosa cells, respectively due to the reaction of the substrate AcSCh with the enzyme present in cells..

In Treatment 3 in addition to the staining solution and the substrate acetylthiocholine (AcSCh) there is eserine hemisulfate, an inhibitor of the esterases. As we expected no colour was observed in cells as shows Figure 20-c for RTG-2 and Figure 36-c for granulosa cells. This indicates us that the entire colour produced in Treatment 2 is due to the activity of esterases present in both types of cells.

Since we observed that there was enzyme activity in both cells we proceeded to perform the enzyme characterization following the same method than authors like Rendon-von Osten et al., 2005; Nunes et al., 2003; Varó et al., 2001; García et al., 2000, Sturm et al., 1999). Firstly, we assessed the substrate specificity of the enzyme using three substrates: Acetylthiocholine (AcSCh), Propionylthiocholine (PrSCh) and Butyrylthiocholine (BuSCh) in order to optimize the AChE activity measurements in cells. For RTG-2 and granulosa cells, the highest activity was found with AcSCh. A very low activity was measured for PrSCh and BuSCh (Figure 21 and 37 for RTG-2 and granulosa cells, respectively). The effect of substrate concentration is also shown in Figure 21 and 37. For the tested concentrations, AChE activity shows an apparent Michaelian behaviour. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot). The apparent Km values for the substrate AcSCh were found to close for the two types of cells: 21 and 21.7 μM respectively for RTG-2 and granulosa cells. The maximum rate of hydrolysis, Vmax, was achieved at 1760 μM and 3153 μM for RTG-2 and granulosa cells respectively (Figure 1 and 2 from the annex 1 and 2 of results). Inhibition of enzymatic activity was observed at the highest concentration of AcSCh tested, 2.56 mM for RTG-2 and 5.12 mM for granulosa cells. (Figure 1 and 2 from the annex 1 and 2 of results).

Secondly, we used three enzyme inhibitors: Eserine hemisulfate, BW284C51 and iso-OMPA in order to characterize the enzyme.
Eserine hemisulfate, an inhibitor of all the esterases, significantly inhibited the enzyme activity in RTG-2 (Figure 22) and granulosa cells (Figure 38) after an incubation period of 45 min. Inhibition by eserine hemisulfate is almost complete at the highest concentration tested (0.1 mM), 88% in RTG-2 and 85% in granulosa cells. Similar results were found for BW284C51, a specific inhibitor of acetylcholinesterases, in RTG-2 (Figure 24) and granulosa cells (Figure 40) also after an incubation period of 45 min.

BW284C51 causes an 80% AChE inhibition in RTG-2 and a 79% inhibition in granulosa cells at concentration of 0.1 mM. In contrast, RTG-2 and granulosa cells were relatively insensitive to iso-OMPA, an inhibitor of butyrylcholinesterases, since it only causes a 31.43% inhibition in RTG-2 and 40.63 % in granulosa cells.

After the enzyme characterization we performed the enzyme determination. The acetylcholinesterase 20%, 50% and 80% inhibition values IC20, IC50 and IC80, respectively and its 95% confidence intervals are shown in Table 4 and Table 8, for RTG-2 and granulosa cells, respectively. The unexposed cells showed an AChE activity of 1.9 nmol/min/mg protein (RTG-2) and 3 nmol/min/mg protein (bovine granulosa cells). Marinovich et al., 1996 found in the human neuroblastoma cell line, SH-S5Y an activity of 13 nmol/min/mg protein. Parran et al., 2005 reported an activity of 6 nmol/min/mg protein in bovine macrovascular endothelial cells (BMECs). Barata et al., 2004 measured the AChE activity in whole Dafnia tissues obtaining a value of 3.5 nmol/min mg protein. These values are lower compared to fish AChE activities. In unexposed Danio rerio we obtained an AChE activity of 115 nmol/min mg prot in head, 218 nmol/min mg prot in muscle and 30 nmol/min mg prot in liver. Other authors found similar activities in fish; Rendón-von Ostén reported 225 nmol/min mg protein in muscle and 57 nmol/min mg protein in head of Gambusia yucatana.

Although chlorpyrifos is less cytotoxic than carbofuran, the AChE inhibition is higher than the produced by carbofuran. Chlorpyrifos causes the 50% inhibition of AChE activity at 1.04 μM in RTG-2 and 20.96 μM in granulosa cells. In contrast, carbofuran causes the 50% inhibition of AChE activity at higher concentrations, 31.44 μM in RTG-2 and 53.78 μM in granulosa cells.

There is a relation between cytotoxicity and AChE inhibition. In the case of RTG-2 cell line, the AChE inhibition caused by chlorpyrifos is higher than cytotoxicity IC50=1.04 μM versus CI50= 35.94 μM (Table 14). It indicates that AChE is a good biomarker of sublethal effects, because whereas there is no cytotoxic effect observed, the AChE inhibition is very important, showing cellular damage (Figure 25). Regarding as granulosa cells, the IC50=20.96 μM and CI50= 36.2 μM values for chlorpyrifos (Table 14) are more equal. In figure 41, we can see that AChE inhibition increase at the same concentrations than cytotoxicity.

On the other hand, the cytotoxic effect produced by carbofuran is highest than AChE inhibiton in both types of cells, CI50= 19.36 μM and IC50= 31.44 μM for RTG-2 and CI50= 23.05 μM; IC50= 53.78 μM; for granulosa cells. It seems that AChE inhibition occurs only when cytotoxic effect is observed (Table 14 and figures 25 and 41).

Otherwise, the sum of individual chlorpyrifos and carbofuran TU's was higher than 1, it suggests that these pesticides in mixture induce antagonistic effects on AChE activity. The IC50 values for the mixture chlorpyrifos-carbofuran are 32.42 μM in RTG-2 and 54.47 μM in granulosa cells (Table 4 and 8, respectively). These values are more similar to carbofuran values than chlorpyrifos values.
It is well known that carbamate and organophosphorus inhibit AChE activity. Mayerhofer et al., 2003 reported that isolated granulosa cells produce acetylcholine, granulosa cells also contain ACh-receptors of the muscarinic subtype (MR), namely M1 and M5 (Mayerhorfer et al., 2003; Fritz et al., 2001), therefore we might suppose that the inhibition of acetylcholinesterase, causes an increase of acetylcholine and via muscarinic M1/M5 receptors produces an increase of intracellular Ca2+ levels that likely affect Ca2+-dependent ion channels and consequently the membrane potential and it is inversely related to cell proliferation (Fritz et al, 2001).

Furthermore, in the present study, we investigate how two cell types originating from different species, fish and mammalian, rainbow trout Oncorhynchus mykiss and cows Bos taurus, respectively differ in their genotoxic response toward genotoxic substances, and if possible differences correlate with differences in the metabolic enzyme of the two cell types.

The genotoxicity of many xenobiotics is influenced by their cellular metabolism. Therefore, differences between cell lines with respect to biotransformation activity or DNA repair capacity might greatly influence the outcome of in vitro genotoxicity tests (Hartmann and Speit, 1995; Speit et al., 1994; Hasspieler et al., 1997).

At 50 pM of chlorpyrifos in RTG-2 cell line the percentage of DNA migration or comets after an exposure of 48 h is about 9% and only 3% in granulosa cells (Figure 26 and 42). At 5 nM of chlorpyrifos the percentage is similar in both types of cells (12 and 14%, respectively). There is no statistical differences using ANOVA $p<0.05$. That indicates that both types of cells are equal sensitive to chlorpyrifos.

At 50 pM of carbofuran the percentage of comets in RTG-2 cells is 5%, whereas in granulosa cells is lower (2%). However, at higher concentrations, such as 1 nM the same percentage of DNA comets is observed in both cells (10-12%). There is no statistical differences using ANOVA p<0.05 (Figure 27 and 43). That indicates that the genotoxic effect of carbofuran is the same in both types of cells.
Therefore, both types of cells have similar sensitivity respect to the percentage of DNA comets.
Moreover, we observed that chlorpyrifos is less genotoxic than carbofuran in both types of cells. It is in concurs with the obtained results of cytotoxicity where we observed that carbofuran is more cytotoxic than chlorpyrifos in both cells as stated above.

In this work we also measured the aromatase activity. The induction of P450 cytochromes (CYPs) is considered to be one of the most sensitive and subtle biochemical cell responses, and generally takes place at much lower doses of a given chemical than those known to cause lethal or general toxic effects (Denison and Whitlock, 1995).

Aromatase is a key CYP (CYP17A1/19A1) in the production of estrogens, catalysing the conversion of androgens, androsteneidone and testosterone via three hydroxylation steps to estrone and estradiol (Brodie et al., 1999; Martucci and Fishman, 1993). Aromatase is expressed in many tissues, including the ovaries, testis, placenta, brain, adipose tissue of the breasts, abdomen, thights and buttocks, and bone osteoblasts.

In granulosa cells the CYP19A1 is high expressed and it may exert a major role in steroidogenesis activation P4 (18-36 ng/n.cell) for low toxic concentration that in bioactivation with a consequent less cytotoxicity.

*RTG-2 cell line presents very low aromatase activity. Progesterone (P4) production is lower than 7 ng/ml/n.cell*100 (Figure 28 and 29). In contrast in granulosa cells we found that P4 production is about 18-36 ng/ml/nºcell and tends to increase with the toxic concentrations (not statistically significant) (Figure 44 and 45). It seems there is a modulatory effect of cholinergic stimulation, through muscarinic receptors and progesterone secretion from bovine granulosa cells (Luck, 1990). Further carbamate and organophosphorus are biotransformated by aromatase P450 that have a key role in regulating granulosa cells steroidogenesis activity and consequently hormone production.*

The preliminary results of this study were described in the 46 ETCS International meeting "IN VITRO CYTOTOXICITY MECHANISMS" held in Verona (Italy) by Stacchezzini et al , 2006 "Preliminary evaluation of carbofuran cytotoxicity and steroidogenesis effects on bovine granulosa cells"" and also in the XVI Spanish Congress of Toxicology held in Cáceres (Spain) by Alañón et al., 2005 "Evaluation of the toxicant effects of two neurotoxics in two different cell lines; RTG-2 from fish gonad and granulosa cells from bovine ovary"

On analysis of the data, the RTG-2 cell line was found to be more sensitive than granulosa cells based on IC50 (AChE inhibiton) for chlorpyrifos. But, for carbofuran and their mixture, there were no differences between both types of cells.

The higher levels of aromatase in granulosa cells than in RTG-2 cell line could explain the differences of AChE inhibition between both cells. It is possible that exists a mechanism of detoxification related to progesterone. And for this reason, granulosa cells have a lower inhibition of AChE than RTG-2 cell line.
The lack of progesterone production due to the low levels of aromatase in the in RTG-2 attests to the lower tolerance of these cells to the toxic effects of chlorpyrifos.
Moreover, the fish cells lines which are cultured at temperatures of 20ºC have much slower growth rates than mammalian cells cultured at 37ºC (Gülden et al., 2005). The population doubling times amount to 50 h and more (Segner, 1998).

With regard to cytotoxicity, RTG-2 cell line and granulosa cells have the same sensibility for chlorpyrifos, cabofuran and their mixture. This indicates, as was also stated by Gülden et al., 2005 and Castaño et al. 2003, that there are no general differences in the sensitivity of fish and mammalian cells towards the basal cytotoxic action of chemicals despite some cell specific cytotoxicities.

5.2. IN VIVO ASSAYS

The justification for in vivo studies is derived from the objective of ecotoxicology to evaluate the effects of chemicals on populations and ecosystems. One inconvenient of the in vitro assays is that they only give information about cellular level. In vitro fish cell tests should be able to generate comparable results on relative potency ranking and effect concentrations of toxicants before being accepted as an alternative to in vivo fish bioassays (Senger, 2005).

Nevertheless, efforts should be undertaken to replace the use of fish acute tests, since this is a legal and ethical requirement laid down in the Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes (European Commission, 1986).

In order to reduce the amount of fish that has to be killed for research, emphasis has to be put on the development of alternative fish cell-line bioassays, which may be used to monitor the water quality. Experiments in using in vitro methods have to be compared with studies on fish, in order to correlate the in vitro results to in vivo data. Good correlations have been determined based on the relative ranking order of pollutants to fish cells with their water-borne in vivo toxicity to live fish (Bols et al., 1985; Dierickx and van de Vyver, 1991; Segner and Lenz, 1993; Castaño et al., 1996).

For this reason, in the present work, mortality and inhibiton of acetylcholinesterase were measured in zebra fish (Danio rerio) in order to compare the obtained values with the in vitro results.

We proposed this specie as a test fish for ecotoxicological assays because of its suitability to laboratory conditions. It has been widely used (Segner et al., 2004; Levin et al., 2004, 2003; Alañón et al., 2003; McAllister et al., 2003; Roex et al, 2003; Riva et al., 2001, 2002; Vallés, 2001, López. 1999) and it is recommended by the Organization for Economic Co-operation and Development (OECD).

Despite of rainbow trout is an autochthon fish, we preferred to use zebra fish because it is easier to maintain in adequate laboratory conditions than rainbow trout.

The acute toxicity in Danio rerio expressed as the LC50 96 h value and its 95% confidence limits for chlorpyrifos, carbofuran and their mixture, to adult, zebra fish Danio rerio were shown in Table 9, and were 72.71 μM (57.83-87.99) for chlorpyrifos, 28.88 μM (24.35-33.54) for carbofuran and 32.45 μM (23.29-52.48) for the mixture. Control mortality was zero. Percentages of mortality related to each pesticide concentration are in Table 3 from the annex 3 of results. Antón et al., 1993 on Carassius auratus, obtained similar results; the LC50 96h for carbofuran was 35.70 μM. In contrast, Rendón von Ostén, 2004 reported an LC50 value of 2.87 uM at only 24 h on mosquito fish, Gambussia yucatana, but they used a commercial preparation of carbofuran, Furadan 350L instead of technical carbofuran. Regarding to chlorpyrifos

other authors such as Selvi et al., 2005 reported lower values of LC50 96 h on guppy Poecilia reticulata, of 5.11 μM (4.20-6.0), for chlorpyrifos-methyl and Rendón-von Ostén 2004 reported an LC 50 96h value of 0.242 μ M for a commercial formulation of chlorpyrifos-ethyl, Lorsban 480EM. Therefore, there is a variation between different species and preparation of the pesticides. No data has found about the acute toxicity of the mixture chlorpyrifos-carbofuran.

Observations of behavioral response of Danio rerio were conducted at 1–8 h and every 24 h during the acute toxicity tests. The control group showed normal behavior during the test period. The unexposed fish did not suffer some impact when they were placed into the aquatic medium at the beginning of the bioassays.

The changes in behavioral response started 1 h after dosing. For chlorpyrifos the 0.014 μM (lowest) concentration had similar behavior with the control group. The observed behavioral changes and effects were typical of neurotoxin toxicity: less general activity than control group, loss of equilibrium, erratic swimming and staying motionless at a certain location generally at mid-water level for prolonged periods. Fish exposed to 0.24 μM showed less general activity with occasional loss of equilibrium. Loss of equilibrium becomes more frequent in the 4.5 μM concentration. The 45 μM and 100 μM concentration groups stayed motionless close to the water surface and later fell to the aquarium bottom in an uncontrolled manner. The highest concentration of 200 μM showed all responses at high intensities: the loss of equilibrium, hanging vertically in water, rapid gill movement, erratic swimming, sudden swimming motion in a spiral fashion, after long periods of motionlessness lying down on the aquarium bottom and suddenly starting to move.

The exposed fish to carbofuran suffered a shock after 3 h and swimmed as if they were exhausted. At 4.5 μM of carbofuran they were showing lethargic movements, although in the following days they survived. This effect probably could be due to the fast degradation of pesticide in the aquatic medium, although mortality continued after the initial shock in the aquarium with the higher assayed dose (100 μM). Other authors (Selvi et al., 2005; Antón et al., 1993) had observed this effect.

Thus, our results agree with the view that carbamates are expected to induce toxicity faster than organophosphorous pesticides such as chlorpyrifos since do not need to be bioactivated to be potent inhibitors of AChE (Smith, 1987).

In the mixture chlorpyrifos-carbofuran at 4.5 μM fishes showed less general activity with occasional loss of equilibrium. At 21 μM concentration fishes stayed motionless close to the water surface and later fell to the aquarium bottom in an uncontrolled manner. At 45 μM the mortality was of 90% (Table 3 of the annex 3 of results). The sum of TUs was lower than 1 (Table 7 of the annex 6 of results). It suggests that these pesticides in mixture induce synergistic effects on fish mortality.

At the end of the acute toxicity assay (96h) the effects on the enzyme acetylcholinesterase (AChE) were analyzed in the surviving fish. The enzyme activities of different tissues, head, muscle and liver were determined with three substrates: Acetylthiocholine (AcSCh), Propionylthiocholine (PrSCh) and Butyrylthiocholine (BuSCh) in order to optimize the AChE activity measurements.

AChE activity expressed in D.rerio head, muscle and liver as a function of increasing concentrations of AcSCh, PrSCh and BuSCh is showed in Figure 46, 47 and 48, respectively. The maximum specificity was obtained with AcSCh in the three tested tissues. Lower activities were observed when PrSCh and BuSCh were used as substrates. Inhibition of enzymatic activity was observed at the highest concentration of AcSCh tested (5.2 mM). For the tested concentrations, AChE activity shows an apparent Michaelian behaviour. The Michaelis constant was calculated using the graphic method of Ligand Binding, one site saturation (Sigma Plot). The apparent Km values for the sustrate AcSCh were 0.198 mM in head, 0.211 mM in muscle and 0.165 mM in liver. The maximum rate of hydrolysis, Vmax, was achieved at 178 mM in head, 577 mM in muscle and 18.5 mM in liver (Figure 3, 4 and 5 from the annex 3 of results).

After determination of substrate specificity, we used three enzyme inhibitors: Eserine hemisulfate, BW284C51 and iso-OMPA in order to characterize the enzyme.
Eserine hemisulfate, an inhibitor of all the esterases, inhibited AChE activity in head, muscle and liver. Figure 49, 52 and 55, respectively after an incubation period of 45 min. Similar results were found for BW284C51, a specific inhibitor of acetylcholinesterases, in head, muscle and liver Figure 51, 54 and 57, respectively. Inhibition by eserine hemisulfate was almost complete at the highest concentrations tested in head (97%), muscle (98%) and liver (95%) samples. BW284C51 caused an AChE inhibition of 99.9% in head and muscle and 99.5% in liver at concentrations of 0.1 mM. In contrast, Danio rerio head, muscle and liver AChE were relatively

insensitive to iso-OMPA, an inhibitor of butyrylcholinesterases it caused only an AChE inhibition of 30% in head, 16% in muscle and 15.5% in liver.

The almost full inhibition of both head (97%), muscle (98%) and liver (95%) AChE by eserine hemisulphate at 0.1mM indicate that the measured activity is predominantly from ChE and not from other esterases. Furthermore, almost full inhibition occurred with BW284C51 while little inhibition was observed with iso-OMPA. Thus, it seems that the main form present in both muscle, head and liver of this specie is AChE. These results are also in good agreement with those found in Gambusia yucatana (Rendón-von Ostén, 2004), Poecilia reticulata (Garcia et al., 2000), in Gambusia holbrooki (Nunes et al., 2003) and, in marine teleost fish such as Limanda limanda, Platichthys flesus and Serranus cabrilla (Sturm et al., 1999).

Once the enzyme characterization was performed, we proceeded to do the enzyme determination. The IC50 values and its 95% confidence limits in head of Danio rerio were 0.81 μM (0.2-2.59) for chlorpyrifos, 43.23 μM (38.2-50.77) for carbofuran and 22.72 μM (16.6-32.7) for the mixture. In muscle were 1.53 μM (0.48-4.64) for chlorpyrifos, 47.65 μM (38.15-78.77) for carbofuran and highest than the maximum concentration tested (45 μM) for the mixture. In liver, were 2.71 μM (1.16-6.03) for chlorpyrifos, 57.34 μM (38.18-167.7) for carbofuran and 32.45 μM (23.3-52.48) for the mixture (Table 10-b).

We observed that chlorpyrifos inhibits much more AChE activity than carbofuran and mixture. In contrast, carbofuran is more toxic than chlorpyrifos. The same results are obtained for RTG-2 cell line and granulosa cells in which the toxicologic interactions of the mixture have a synergistic effect on cytotoxicity, but anatgonistic effect on AChE inhibition.

Regarding the effects of the tested pesticides on AChE activity, both chlopyrifos and carbofuran inhibited the activity of the enzyme in both head, muscle and liver samples. AChE activities of muscle and head of zebrafish were significantly inhibited by chlorpyrifos. LC50 value for chlorpyrifos is one or two orders of magnitude higher than IC50 values. LC50 for chlorpyrifos is 72.71 μM, whereas IC50 was 0.81 μM in head, 2.71 μM in muscle and 1.53 μM in liver, suggesting that the fish died due to AChE inhibition. At 45 μM concentration of chlorpyrifos the percentage of mortality was 20%, while the percentage of AChE inhibition was 84 % in head, 76 % in muscle and 75 % in

liver (Table 3 from the annex 3 of results). This may indicate that some fish (but not all) are able to survive at least for short periods with considerable levels of AChE inhibition. A considerable AChE inhibition (80%) was also found in the mosquitofish (Gambusia affinis) that survived to an exposure of 72 h to chlorpyrifos (Carr et al., 1997). Similar have been found for other organophosphates and other fish, including the sea bass (Dicentrachus labrax) (76% ChE inhibition) after a 96 h exposure to dichlorvos (Varó et al., 2001).

Comparing these values to the in vitro results we observed the same effect in RTG-2 cell line, the CI50, concentration that causes a cytotoxicity of 50%, is 35.9 μM and IC50, concentration that inhibits the AChE 50%, is 1.04 μM, a value very similar to the obtained in head of zebrafish (0.81 μM).
In contrast, in granulosa cells there are no differences statistically significants between the CI50 (36.2 μM) and IC50 (20.9 μM) values (Table 14).

On the other hand, LC50 value for carbofuran were the half of IC50, LC50 was 28.88 μM and IC50 was 43.23 μM in head, 57.34 in muscle and 47.65 μM in liver. It is strange, because whereas the mortality is elevated the AChE inhibition is the half. At 45 μM of carbofuran the mortality was 80% but the AChE inhibition was only 49% in head, 53% in muscle and surprisingly there was no inhibition in liver. It could suggest that mortality is due to another cause than AChE inhibition or that the inhibition of AChE produced by carbofuran has a temporal effect different that the permanent effect of chlorpyrifos. It is known that carbofuran produces a reversible inhibition of AChE, maybe for this reason we cannot detect the complete and real AChE inhibition caused by carbofuran, because its recovery is very fast and likely the AChE inhibition was understimated due to a partial reactivation of AChE inhibited by carbofuran.

We observed the same case in RTG-2 cell line, CI50 is 19.3 μM and IC50 is 31.4 μM and also in granulosa cells, CI50 23.05 μM and IC50 53.78 μM (Table 14).

Also Barata et al., 2004, found that in Daphnia magna juveniles the lethal effects of chlorpyrifos occurred at high 50–80% AChE inhibition and in contrast the lethal effects of carbofuran occurred at low 20–40% AChE inhibition levels.

In eels Fernández-Vega et al. (2002) also found an inhibition of 30–50% of brain and muscle AChE after 2 h exposure to the carbamate thiobencarb.

Studies in mussels reported that the relative sensitivities of B-esterases varied across carbamates (Galloway et al., 2002).

On the other hand, the effects of a 40% decrease in AChE activity in zebrafish are difficult to estimate. Weiss (1958, 1961) reported that AchE inhibition as low as 8% is lethal to some fish species, whereas other studies have reported that some fish are able to survive inhibition of 70–90% (Weiss 1961; Coppage and Matthews 1974; Richmonds and Dutta, 1992). Zinkl et al. 1991 reported that greater than 70% AChE inhibition in fish commonly results in death. At 20–70% inhibition, adverse effects become more subtle and can include reproduction problems, and alterations in behavior (Beyers and Sikoski, 1994).
Therefore, there is some controversy in the literature relative to the extent of AChE depression required to cause death in aquatic animals. In studies with aquatic invertebrates including Daphnia, AChE inhibition after exposure to lethal concentrations of anti-AChE was usually in the range of 70–100% (Barata et al., 2001; Bocquené et al., 1991; Detra and Collins, 1991).
In same cases, however, lethal effects of anti-AChE occurred at an AChE inhibition of 40% or below (Escartín and Porte, 1996a; Bocquené et al., 1991).

In the study of Barata et al., 2004, the AChE of Daphnia magna juveniles inhibited by organophosphorous pesticides took 24 and 96 h to recover 50% and almost 100%, respectively, whereas complete recovery of AChE to carbofuran was achieved within the first 12 h. The fact that recovery enzymatic rates were slower for organophosphorous pesticides than for carbamates is not surprising since organophosphorous pesticides are considered irreversible inhibitors of AChE when compared with carbamate pesticides, thus reactivation of inhibited enzyme takes place very slowly in comparison with AChE inhibited by carbamates (Smith, 1987). Indeed in most cases AChE inhibited by organophosphorous pesticides can only be recovered by de novo synthesis of the enzyme (Boone and Chambers, 1996). Reported studies in aquatic crustacea and fish species indicate that AChE inhibited by organophosphorous pesticides took from 2 to >4 weeks to recover to control levels (Kuhn and Streit, 1994; Mc Henerey et al., 1996; Escartín and Porte, 1996b; Boone and Chambers, 1996).

Different species may differ considerably in their sensitivity to toxicants and in the same environment closely related species can display very different exposure due to behaviour, food preference or life stage (Chapman, 1981) and it is not possible to know

a priori which step is the most sensitive or the 'key reaction' (Cairns and Pratt, 1989; Cairns and Niederlehner, 1987; Cairns, 1986).

Communities and ecosystems are characterized by a complex network between organisms interacting with each other and with the abiotic environment. In single-species toxicity testing such interactions are not taken into consideration. It the single-species approach ignores the importance of information on interactions among individuals and among species and that it does not recognize that the behaviour of a large-scale system generally cannot be predicted from the behaviour of its subunits.

The use of multi-species laboratory test systems could provide a possible way to detect effects that depend on interactions between species. In this work apart from the zebra fish Danio rerio we also used the freshwater algae Chlorella vulgaris, Selenastrum capricornutum and Scenedesmus subspicatus and the luminescent bacterium Vibrio fischeri.

Different Environmental Protection Agencies (USA, Canada, and China) recommend the green algae Scenedesmus subspicatus, Selenastrum capricornutum and Chlorella vulgaris for acute tests as ecological indicators because of their high sensitivity to contaminants. They have been used in a lot of studies (Ma et al., 2006, 2005, 2002; Bengtson et al., 2005; Mukherjee et al., 2004; Alañón et al., 2003; Gibert et al., 2002; Riva et al., 2001, 1992; Vallés, 2001, Moreno-Garrido et al., 2000).

Moreover, according to the OECD Test Guideline 201, AFNOR T90-304 and EEC (1988) protocols, these algae are suitable species to study the effects of chemicals on their growth population.

On the other hand, methods for conducting aquatic toxicity tests as described in various guidelines, including those of the OECD, EPA, EU, and ISO, are typically designed for substances that are essentially pure, readily water soluble, and chemically stable. As a consequence, when such methods are applied to unstable or complex substances, or sparingly soluble substances that do not display toxicity at the solubility limit, difficulties in conducting and interpreting toxicity tests are encountered (Rufli et al., 1998). Most pesticides are poorly water soluble and low solvent soluble. The pesticides tested were dissolved with a little ethanol. The concentration of solvent in medium was kept at a minimum. Most were the lower maximum allowable limit of 0.05% solvent for acute tests, which is recommended by the U.S. Environmental Protection Agency; this level was not significant with respect to toxicity (Jay, 1996).

In this work, carbofuran, chlorpyrifos and their mixture have been tested to examine their effects on the green algae Scenedesmus subspicatus, Selenastrum capricornutum and Chlorella vulgaris, and then compared for their differential sensitivities. The 72 h EC50 values for the green algae are reported in Table 11-b. Scenedesmus subspicatus is the most sensitive to chlorpyrifos, with EC50= 71.21 μM, between Chlorella vulgaris and Selenastrum capricornutum there is no statistical differences with an EC50 of 127.5 and 132.4 μM, respectively. In the case of carbofuran the most sensitive is Chlorella vulgaris with EC50 value of 27.04 μM. There is no statistical differences between Scenedesmus subspicatus EC50=56.81 and Selenastrum capricornutum EC50= 62.93 μM.

The same effect happens for the mixture chlorpyrifos-carbofuran. Chlorella vulgaris is the most sensitive EC50=24.94 μM, and there is no differences between Scenedesmus subspicatus EC50=51.33 μM and Selenastrum capricornutum EC50= 62.93 μM.

Therefore we observe variations in response to pesticides among individual species of the green algae. Selenastrum proved to be more tolerant genera, whereas Chlorella is more sensitive for carbofuran and the mixture but not for chlorpyrifos. Scenedesmus is the most sensitive to chlropyrifos.

The effect of chlorpyrifos and carbofuran is very much species specific, Selenastrum capricornutum is the less sensitive specie. Sensitive varies not only among pesticides, but also among species. There are no species that could be identified as ''always being the most sensitive or always the least sensitive'' (Wang and Freemark, 1995). Similar results were obtained in a study of Ma using 42 herbicides as test compounds (Ma et al., 2001; Ma and Liang, 2001; Ma, 2001).

Investigations using different algal species as test organisms have shown that algae vary greatly in their response to chemicals (Ma et al., 2006; 2004, 2002; Vallés, 2001; Sáenz et al., 1997). Differential sensitivity of green algae to the compounds could induce species shifts within communities (Tadros et al., 1994; Boyle 1984).

Acute toxicity was also measured in the marine bacterium Vibrio fischeri. The bioluminescence of V. fischeri is inhibited by toxicants. To monitor the inhibition, different dilutions of the pesticides were mixed with the bacterial suspension. The decline in light emission was measured after contact periods of 5 and 15 min in separate experiments.

The selection of pesticide concentrations was limited by the fact that the Microtox test requires that the compound be soluble and stable in 2% (w/v) saline solution. The initial

experiments determined the amount of bioluminescence inhibition caused by specific concentrations of the test chemicals. The initial concentrations of the pesticides were then modified in order to obtain concentrations in which, after the dilution steps indicated by the Microtox Basic procedure, the amount of pesticide which reduced the bioluminescence of Vibrio fischeri by about 50-60% was in the intermediate zone of the graphic dose-response, where the confidence intervals at 95% of the EC50 values were minimum.

An important limitation of the Microtox system is that only the toxicity of aqueous solutions can be determined. Addition of organic solvents to increase the solubility of chemicals resulted in increased toxicity to Vibrio fischeri. However, an organic solvent is normally used to dissolve substances with minimal water solubility. Several, like ethanol (Tay et al. 1992) or dimethylsulfoxide (Dutka et al. 1991; Kwan and Dutka 1990), have been studied, but we used ethanol because is the solvent used in the others tests. According to the data obtained by different authors, the percentage of the solvent in the test solution should not be greater than 10%. Recommended percentages include 4% (Kwan and Dutka 1990), 5% (Kaiser and Palabrica 1991), 8% (Somasundaram et al., 1990) and 10% (Kaiser and Ribó, 1987). We checked that concentrations of 8% ethanol were not toxic to the bacteria.

The EC50 values at 5 and 15 min and its 95% confidence limits are reported in Table 12. Carbofuran (EC50 5 min= 63.58 μM; EC50 15 min= 56.10 μM) is more toxic than chlorpyrifos (EC50 5 min=212.65 μM; EC50 15 min= 195.79 μM). Similar results were reported for carbofuran by Fernández-Alba et al., 2001 EC50 5 min=44.29 uM and at 15 min 43.38 μM, Somasundaram et al., 1990 EC50 5 min= 92.65 μM and Ruiz et al., 1997, EC50 15 min =108 μM. For chlorpyrifos, EC50 (5 min) reported by Somasundaram et al, 1990 was 132 μM.

Therefore, the reproducibility of the Microtox test is better than 25%. Previous interlaboratory studies have shown a satisfactory reproducibility for the Microtox assay. These results were presented by Ribó et al. 2005 at the 18th Annual meeting of SETAC Europe 2005. "Variability of results for mixture toxicity with the luminiscent bacteria bioassay" in Lille (France).

On the other hand, looking at the toxicity values we observed that the toxicity increases with the time. The EC50 values declined with increasing duration of exposure (5, 15

min) to pesticides. EC50 values for 5 min incubation are greater than for 15 min incubation (Table 12). This is understandable, as a larger amount of toxicant is needed to produce the same response over a shorter incubation period. In Danio rerio (96 h of exposure) and the freshwater algae (72 h of exposure) the toxicity value is little than Vibrio fischeri (5, 15 min of exposure) (Table 14) indicating also that toxicity increases with the time. Figures 62 a-c show double log plots of the gamma (Γ) function versus concentration of chlorpyrifos (a), carbofuran (b) and their mixture (c). Light emission or bioluminiscence declined and, correspondingly, percent inhibition increased with increasing concentrations of pesticide.

Whole organisms assays based on single prokaryote, such as V. fischeri, are not sufficiently representative of possible toxicological impact of a compound at various levels of the ecosystem. Laboratory toxicity studies generally focus on the effects of a single toxicant on an organism. In natural aquatic systems, organisms are exposed to combinations of chemicals and other potential stressors. The effects of stressors can combine in an additive, a less than additive (antagonistic), or a greater than additive (synergistic) manner. Therefore, an understanding of the interactions between stressors is necessary to determine the proper application of laboratory studies to the natural environment (Herbrandson et al., 2003).

In mixture experiments, a toxic units (TU) approach was used to establish toxicology interactions. TUs were calculated by taking the amount of each chemical at the EC50 of the mixture and dividing it by the individual pesticide EC50 value. The value of the TUs sum will indicate the type of interaction: if TUA+TUB>1 the joint action is antagonistic; if TUA+TUB=1 the joint action is additive; if TUA+TUB<1 the action is synergistic. Table 7 from the annex 6 of results shows the TUs values obtained in each assay. When using pesticides mixtures, the concentration of the toxicant was defined as the sum of concentrations of all the pesticides in solution. Mixtures of pesticides were prepared by combining various stocks solutions of pure components in 1:1 ratio. Therefore, pesticides in the mixtures were at the same concentration used when tested singly.

In all the tested species there is a synergistic effect caused by the mixture chlorpyrifos-carbofuran. In contrast, chlorpyrifos and carbofuran when tested single inhibited acetylcholine esterase (AChE) activity but when tested in mixture showed a toxicity interaction of antagonisms on AChE activity.

Conclusions

6. Conclusions

1.- In both in vitro assays using cells (RTG-2 fish cell line and bovine granulosa cells) and in all the tested organisms (Danio rerio, Chlorella vulgaris, Scenedesmus subspicatus, Selenastrum capricornutum and Vibrio fischeri), carbofuran is more toxic than chlorpyrifos. The ranking of toxicity (based on EC50 values) of the tested pesticides was carbofuran>chlorpyrifos.

2.- In contrast, chlorpyrifos inhibits much more the enzyme acetylcholinesterase in all the Danio rerio tissues, head, muscle and liver, in RTG-2 cell line and bovine granulosa cells. The ranging of toxicity (based on IC50 values) was chlorpyrifos>carbofuran.

3.- The RTG-2 cell line and bovine granulosa cells were the most sensitive assay. For the most part, Vibrio fischeri was the less sensitive of the used assays with the exception of their response carbofuran. In that case, Selenastrum capricornutum was the less sensitive.

4.- *Synergistic and antagonistic toxicity effects were observed with pesticide cocktails. The joint action of chlorpyrifos and carbofuran is synergistic regarding acute toxicity but antagonistic regarding AChE inhibition*

5.- The differential responses both in cytotoxicity, genotoxicity and inhibiton of AChE values demonstrate that a unique assay is not enough. A single bioassay will never provide a full picture of the quality of the environment. A battery of test systems and indicators representative of a wide range of organisms should be used. A minimum ecotoxicological in vitro test battery should at least include bacteria, algae, invertebrates and cell lines.

6.- Generally, data from in vivo bioassays have greater ecotoxicological relevance because they represent an integrated biological response. However, animal toxicity testing is costly and requires the sacrifice of many organisms. In relation to this, the use of fish cells in culture for in vitro cytotoxicology assays of environmental samples constitutes an alternative to acute toxicity testing using laboratory fish populations.

7.- The in vitro assays, which are rapid, reproducible, simple and inexpensive, clearly show the effects of organophosphorous and carbamates pesticides on fish and mammalian cells suggesting the potential impact of these pesticides on organisms in vivo.

8.- There are limitations associated with the in vitro systems. In vitro bioassays do not account for the biokinetics, tissue distribution and biotransformation that may occur in vivo and although in vitro assays may not always reflect the true in vivo situation, but they undoubtedly provide important data on the activity of different compounds at the cellular level. At this level, the data can be utilized to better understand the acting mechanism of the toxic as activator/inhibitor of enzymatic activities such as AChE and aromatase

9.- The differential sensitivities demonstrated by cells used in this study show that they could be exploited as screening tools (Tier 1 assessment) to rank environmental samples in order of pollution status.

10.- This research also highlights the influence of study design (e.g. test duration), bioassay and endpoint selection. In conclusion, the use of a battery style approach is recommended, as it affords a holistic appraisal of the ecotoxicological assessment of potential contaminants.

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Annex

ANNEX-MATERIALS AND METHODS

ANNEX 1

1.1 CELL CULTURE MEDIA

Both primary bovine cells and continuous fish cell lines are incubated in mammalian culture media. While primary cells are often maintained in serum-free media, media for continuous fish cell lines are usually supplemented with mammalian serum, although, in some cell lines, the addition of more-defined supplements such as insulin may be adequate.

Because of animal welfare and ethical considerations, the replacement of calf or foetal bovine serum is requested. However, the presence of mammalian serum may influence the physiology of the cells and their toxicological responses. Serum may alter the bioavailability of chemicals tested (Hesterman et al., 2000; Fent, 2001).

For this reason we tested two diferent media with 10% and 1% of foetal calf serum prior to the experiments.

TABLE 1. *Cell medium composition. All the reactives were purchased from Biochrom.*

MEDIUM MEM (M 1)	**MEDIUM MEM (M 3)**	**MEDIUM DMEM (DM 1)**	**MEDIUM DMEM (DM 3)**
MEM EAGLE (Minimum essential medium with Earle salts)	*MEM EAGLE (Minimum essential medium with Earle salts)*	*DMEM EAGLE + F12 (Dulbecco modified essential medium)*	*DMEM EAGLE + F12*
NEAA (Non Essential Aminoacids) 1%	*NEAA (Non Essential Aminoacids) 1%*	-	-
Foetal calf serum (FCS) 10%	*Foetal calf serum (FCS) 1%*	*Foetal calf serum (FCS) 10%*	*Foetal calf serum (FCS) 1%*
Antibiotics Penicillin/streptomycin 1%	*Antibiotics Penicillin/streptomycin 1%*	*Antibiotics Penicillin/streptomycin 1%*	*Antibiotics Penicillin/streptomycin 1%*
Amphotericine 1%	*Amphotericine 1%*	*Amphotericine 1%*	*Amphotericine 1%*
L-glutamine 1%	*L-glutamine 1%*	*L- glutamine 1%*	*L- glutamine 1%*
-	-	*Insulin, trasferrin, sodium selenite (ITS) 1%*	*Insulin, trasferrin, sodium selenite (ITS) 1%*
-	-	*Sodium pyruvate 1%*	*Sodium pyruvate 1%*

No growth differences were observed between Minimum essential medium (MEM) and Dulbecco modified essential medium (DMEM) and between 10 and 1% FCS, so for all the culture the cell media used was MEM (Minimum essential medium) 10% and for the experiments MEM 1%.

ANNEX 2

2.1. ATP DETERMINATION

The bioluminescent determination of the Adenosine 5'-triphosphate (ATP) released from a suspension of viable cells was evaluated. Estimates of cell concentrations may be calculated if it is assumed that the ATP content per viable cell remains fairly constant. The number of viable cells is selectively counted because, as a cell dies, its ATP is rapidly degraded (Lundin, 1982).

Very generally, viable cells will contain about 1 picogram (10^{-12} gram) or approximately 2 femtomoles (2×10^{-15} moles) of ATP per cell (Harber, 1982). Viable somatic cell ATP may be determined as follows:

Intracellular ATP $\xrightarrow{FLSAR}$ *Free ATP (1)*

Free ATP + Luciferin $\underset{Mg^{++}}{\overset{Firefly\ Luciferase}{\rightleftharpoons}}$ *Adenyl-luciferin + Ppi (2)*

Adenyl-luciferin $+ O_2 \rightarrow$ *Oxyluciferin + AMP +* CO_2 *+ Light (3)*

Reactions (1) and (3) are essentially irreversible and Reaction (2) is reversible with the equilibrium lying far to the right (Strehler, 1974). When ATP is the limiting reagent, the light emitted is proportional to the ATP present, which is in turn proportional to the number of somatic cells in the sample.

We used the kit Bioluminiscent somatic cell assay kit purchased from Sigma-Aldrich (St. Louis, MO, USA). This kit can measure the ATP released by fewer than 10, or as many as 2×10^5 viable somatic cells (or a sample containing from 400 to 8×10^6 cells per ml). This compares very favorably with the sensitivity obtained using a microscope with a hemocytometer, which may only detect down to about 2×10^5 cells per ml. The results obtained with this kit will depend mainly on the amount of quenching by the media in which the cells are suspended.

2.1.1. REACTIVES

ATP ASSAY MIX. The ATP Assay Mix is a lyophilized powder containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts. It is stable indefinitely if stored desiccated below 0ºC and protected from light.

ATP ASSAY MIX DILUTION BUFFER. The ATP Assay Mix Dilution Buffer is a lyophilized powder containing $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts. It is stable indefinitely if stored desiccated below 0ºC.

ATP STANDARD. The ATP Standard is a preweighed lyophilized powder. Each vial contains approximately 1.0 mg (2.0×10^{-6} mol) of Adenosine 5'-triphosphate. Actual ATP content in vial is given on product label. Store desiccated below 0ºC.

SOMATIC CELL RELEASING REAGENT. The Somatic Cell Releasing Reagent is a concentrated preparation which increases membrane permeability to many small molecules. Cellular ATP is released almost immediately. This reagent is stable indefinitely at 0-5ºC.
All components of the kit were reconstituted in ultrapure sterile-filtered distilled water.
All assay vials; glassware and pipet tips were cleanedwith a soaking in 1 N HCl overnight followed by a thorough rinse in deionized water in order to be free from ATP and bacterial contamination. We used polypropylene tubes due to the absence of self-fluorescing impurities.

The content of one vial of ATP Assay Mix was dissolved in 5 ml of sterile water to generate a stock solution with pH of 7.8. After mix by gentle inversion we allowed the solution to stand in ice for at least one hour to assure complete dissolution. During this time a decrease in background may also be seen. This solution is stable for at least two weeks when stored at 0-5ºC and protected from light.
A slight decrease in light production and sensitivity may occur during this time. The stock solution was dispensed in aliquots and frozen for future use.
A dilution of the ATP Assay Mix approximately 25-fold with ATP Assay Mix Dilution Buffer was done. The light detected will not only depend on the number of cells and the amount of ATP per cell, but also on the degree to which the media suspending the cells quenches the light produced.
The content of one vial of ATP Assay Mix Dilution Buffer was dissolved in 50 ml of sterile water. This solution is stable for at least two weeks at 0-5ºC.

An ATP Standard stock solution was prepared by dissolving the contents of one vial of ATP Standard with 10 ml of sterile water. This solution is stable for at least 24 hours at 0-5ºC or over two weeks at -20ºC. Serial dilutions of ATP Standard stock solution with sterile water were done. These dilutions were stored in ice bath.
The Somatic Cell ATP Releasing Reagent was diluted 10-fold with sterile water before use.

2.1.2. PROCEDURE

100 μL of ATP Assay Mix solution were added to a reaction vial. It was swirled and allowed to stand at room temperature for approximately 3 minutes. During this period any endogenous ATP was hydrolyzed, thereby decreasing the background.
To a separate vial containing 100 μL of Somatic Cell ATP Releasing Reagent, we added 50 μL of phospate buffer (100 mM pH 7.4) and 50 μL of the cell sample.
100 μL was transfered to the reaction vial and immediately measured the emitted amount of light using a Sirius luminometer.

ANNEX 3

3.1. ACETYLCHOLINESTERASE ASSAY

3.1.1. REACTIVES

Phosphate buffer (PBS) 100 mM (pH 7.4, 20ºC) Keep at room temperature

K_2HPO_4 (M=174.18g/mol) (basic) : 17.4 g /L H_2O
KH_2PO_4 (M= 136.09 g/mol) (acid) : 6.8 g/L H_2O

Add 600 ml K_2HPO_4 + 300 ml KH_2PO_4
Adjust to pH 7.4

Acetylthiocoline iodide (M=289.7g/mol) Keep at -20ºC

Dissolve 5.9533 mg in 2 ml H_2O.
Keep in ice and protected from light.
DTNB (M= 396.3g/mol) Keep at room temperature

Dissolve 4.9537 mg in 2 ml de phosphate buffer

Keep in ice and protected from light.

3.1.2. REACTION MIXTURE

1.5 ml acetylthiocoline +1.2 ml DTNB in 30 ml phosphate buffer. Prepared just before use.

All reactives were purchased from Sigma (St Louis MO).

3.1.3. PROTEIN QUANTIFICATION REACTIVES

- BSA (Bovine serum albumine) (4ºC)
Prepare stocks of 1 mg/ml.

- Standards concentrations:
0.2, 0.1, 0.04, 0.02 mg/ml.

-Bradford reagent (4ºC). Protect from light.
6 ml Bradforf reagent + 24 ml H_2O (rate 1:4). Prepare just before analysis.

ANNEX 4

4.1.ACUTE TOXICITY IN MICROALGAE

TABLE 2. *Algal medium according OCDE 201 for the culture of Chlorella vulgaris, Selenastrum capricornutum and Scenedesmus subspicatus.*

Stock 1	
NH_4Cl	*15 mg/l*
$Mg\ Cl_2 \cdot 6H_2O$	*12 mg/l*
$CaCl_2 \cdot 2\ H_2O$	*18 mg/l*
$MgSO_4 \cdot 7H_2O$	*15 mg/l*
KH_2PO_4	*1.6 mg/l*
Stock 2	
$FeCl_3 \cdot 6H_2O$	*0.08 mg/l*
$Na_2EDTA \cdot 2H_2O$	*1 mg/l*
Stock 3	
H3BO3	*0.185 mg/l*
$MnCl_2 \cdot 4H_2O$	*0.415 mg/l*
$ZnCl_2$	$3x10^{-3}$ *mg/l*
$CoCl_2 \cdot 6H_2O$	$1.5x10^{-3}$ *mg/l*
$CuCl_2 \cdot 2H_2O$	10^{-5}*mg/l*
$NaMoO_4 \cdot 2H_2O$	$7x10^{-3}$ *mg/l*
Stock 4	
$NaHCO_3$	*50 mg/l*

The pH of this medium after equilibration with air is aproximately 8.

All reactives were reagent grade and were purchased from Sigma (St Louis MO)

ANNEX 5

5.1. INHIBITION OF BIOLUMINISCENCE OF *Vibrio fischeri*

5.1.1.CALCULATION OF ECx AND THEIR CONFIDENCE LIMITS

The inhibition effect of the different pesticide concentrations was compared with a toxicant-free control to obtain percent inhibition (INH%); thus

$$INH\% = (1 - I_x / \varphi I_0) * 100$$

where I_0 and I_x are the luminescence intensities of the sample initially and after contact time x min, respectively, and φ is a correction factor for the control.
The latter is calculated as follows:

$$\varphi = I_x / I_0$$

where I_0 and I_x are the luminescence intensities of the control initially and after contact time x min, respectively. Inhibition data were expressed in terms of the gamma function, i.e. the ratio of light lost to light remaining

$$\Gamma = [(\varphi I_0 - I_x) / I_x]$$

Gamma is related to percent inhibition as follows:

$$\Gamma = INH\% / (100 - INH\%)$$

The concentration of sample causing a 50% reduction in light was designated the EC50 value (expressed in µM) for the sample.
The linear equation for prediction of concentration from gamma values is:

$$Log\ C = b \log \Gamma + \log a$$

This equation describes a line with a slope of b and an intercept of log a, in which C represents concentration and Γ the corresponding gamma.

Regression statistics of log C on log Γ are used to estimate the concentration, which would give a nominal effect like EC50. Therefore, the EC50 is equal to the antilog of

the intercept (log a). The concentrations that cause other percent effects (%E) can be estimated by substituting the log of the equivalent gamma value in the regression equation.

$$\%E = [\Gamma / (1+\Gamma)] * 100\%$$

Standard statistics are used to calculate confidence intervals for this estimate. The specific equation for a 95% confidence pre-factor (PF) in this case is:

$$PF = \sqrt{[S^2(1/N) + ((\log \Gamma_0 - \log \Gamma_a)^2 / \sum \gamma^2)]}$$

In this equation $\Gamma 0$ is the gamma corresponding to the ECXX of interest, log $\Gamma 0$ is the mean of log (gamma) values, and N is the number of data pairs used for regression.

The residual variance, S2, is calculated from the regression statistics as:

$$S^2 = [\sum c^2 - (\sum c\gamma)^2 / (\sum c\gamma^2)] / (N-2)$$

The product of PF and $t_{0.05}$ (from Student's t table with N-2 degrees of freedom) is added to and substracted from log C_0 to give the logs of the higher and lower 95% confidence limits. The antilogs are then taken of log C_0 and its limits.

ANNEX-RESULTS

ANNEX 1

1.1. CYTOTOXICITY AND AChE INHIBITION IN RTG-2 FISH CELL LINE

TABLE 1. *Percentages of cytotoxicity and AChE inhibition in RTG-2 fish cell line related to the used pesticide concentrations after 48 h of exposure (each value is the mean of 6 replicates for cytotoxicity and two for AChE determination and two experiments).*

CHLORPYRIFOS

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,045	*4,90*	*1,65*	*2,9*	*2,74*	*2,93*
0,1	*10,80*	*8,9*	*8,7*	*8,69*	*25,53*
0,2	*15,66*	*13,68*	*14,59*	*13,33*	*32,98*
1	*19,17*	*16,95*	*20,52*	*16,96*	*49,47*
4,5	*38,25*	*33,85*	*34,8*	*32,57*	*54,79*
10	*47,70*	*43,65*	*48,7*	*42,32*	*78,72*
45	*59,55*	*50,50*	*53,05*	*49,56*	*100,00*
100	*64,50*	*60,70*	*67,6*	*58,28*	*100,00*

CARBOFURAN

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,0045	*2,9*	*2,7*	*1,55*	*2,3*	*0,00*
0,045	*3,45*	*4,85*	*11,2*	*5,4*	*2,93*
0,45	*13,4*	*13,95*	*15,1*	*12,9*	*10,90*
1	*17,9*	*20,13*	*21,2*	*23,2*	*26,80*
4,5	*28,95*	*31,4*	*32,0*	*28,2*	*33,50*
10	*37,45*	*31,15*	*33,7*	*31,0*	*35,00*
45	*70,3*	*50,3*	*61,3*	*54,4*	*42,00*
100	*94,4*	*87,75*	*92,4*	*83,5*	*69,95*

MIXTURE

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,045	*2,25*	*0,5*	*1,65*	*1,2*	*0*
0,1	*4,55*	*1,8*	*2*	*2,5*	*0,8*
0,2	*12,8*	*9,5*	*13,1*	*10,5*	*10,9*
1	*25,2*	*20,85*	*29,9*	*22,3*	*16,22*
4,5	*29,15*	*24,25*	*35,45*	*26,1*	*28,46*
10	*40,1*	*40,65*	*45,6*	*38,2*	*30,85*
45	*63,35*	*56*	*61,2*	*54,7*	*48,14*
100	*88,75*	*75,7*	*88,8*	*76,1*	*68,09*

1.2. Calculation of Michaelis-Menten' s constant for acetylcholinesterase enzime in RTG-2 fish cell line

Substrate : Acetylthiocholine

Nonlinear Regression

[Variables]
x = col(1)
y = col(2)
reciprocal_y = if(y=0;"--";1/abs(y))
reciprocal_ysquare = if(y=0;"--";1/y^2)
[Parameters]
Bmax = max(y) "Auto {{previous: 1,76129}}
Kd = if(x50(x;y)>0; x50(x;y); if(mean(x)=0; 1; abs(mean(x)))) "Auto {{previous: 0,0209765}}
[Equation]
*f = Bmax*abs(x)/(Kd + abs(x))*
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
Bmax>0
Kd>0
[Options]
tolerance = 1e-9
stepsize = 1
iterations=200

R = 0,96778485Rsqr = 0,93660752 Adj Rsqr = 0,92755146

Standard Error of Estimate = 0,1228

	Coefficient	Std. Error	t	P
Bmax	**1,7613**	**0,0634**	**27,7834**	**<0,0001**
Kd	**0,0210**	**0,0041**	**5,1348**	**0,0013**

Analysis of Variance:

	DF	SS	MS	F	P
Regression	*1*	*1,5588*	*1,5588*	*103,4232*	*<0,0001*

Residual	*7*	*0,1055*	*0,0151*
Total	*8*	*1,6643*	*0,2080*

PRESS = 0,1870

Durbin-Watson Statistic = 1,5095

Normality Test: K-S Statistic = 0,2005 Significance Level = 0,8178

Constant Variance Test: Passed (P = 0,5804)

Power of performed test with alpha = 0,0500: 0,9990

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	*1,7470*	*-0,1770*	*-1,4416*	*-1,6633*	*-1,9801*
2	*1,7329*	*-0,0829*	*-0,6752*	*-0,7708*	*-0,7460*
3	*1,7054*	*0,0796*	*0,6484*	*0,7273*	*0,7003*
4	*1,6529*	*0,0921*	*0,7499*	*0,8210*	*0,7996*
5	*1,5571*	*0,1129*	*0,9192*	*0,9863*	*0,9841*
6	*1,3954*	*-0,0154*	*-0,1255*	*-0,1361*	*-0,1262*
7	*1,1554*	*0,1346*	*1,0964*	*1,2682*	*1,3378*
8	*0,8597*	*-0,1397*	*-1,1376*	*-1,3969*	*-1,5229*
9	*0,5686*	*-0,0436*	*-0,3551*	*-0,4176*	*-0,3915*

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	*0,4581*	*0,2488*	*-1,1395*
2	*0,0901*	*0,2327*	*-0,4108*
3	*0,0683*	*0,2051*	*0,3558*
4	*0,0670*	*0,1658*	*0,3564*
5	*0,0736*	*0,1314*	*0,3827*
6	*0,0016*	*0,1500*	*-0,0530*
7	*0,2716*	*0,2525*	*0,7774*
8	*0,4955*	*0,3368*	*-1,0852*
9	*0,0334*	*0,2770*	*-0,2423*

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	*1,7470*	*1,6022*	*1,8918*	*1,4226*	*2,0714*
2	*1,7329*	*1,5929*	*1,8729*	*1,4106*	*2,0552*
3	*1,7054*	*1,5739*	*1,8369*	*1,3867*	*2,0241*
4	*1,6529*	*1,5347*	*1,7711*	*1,3395*	*1,9664*
5	*1,5571*	*1,4519*	*1,6624*	*1,2484*	*1,8659*
6	*1,3954*	*1,2830*	*1,5078*	*1,0841*	*1,7067*
7	*1,1554*	*1,0095*	*1,3013*	*0,8305*	*1,4803*
8	*0,8597*	*0,6912*	*1,0281*	*0,5240*	*1,1953*
9	*0,5686*	*0,4158*	*0,7214*	*0,2405*	*0,8966*

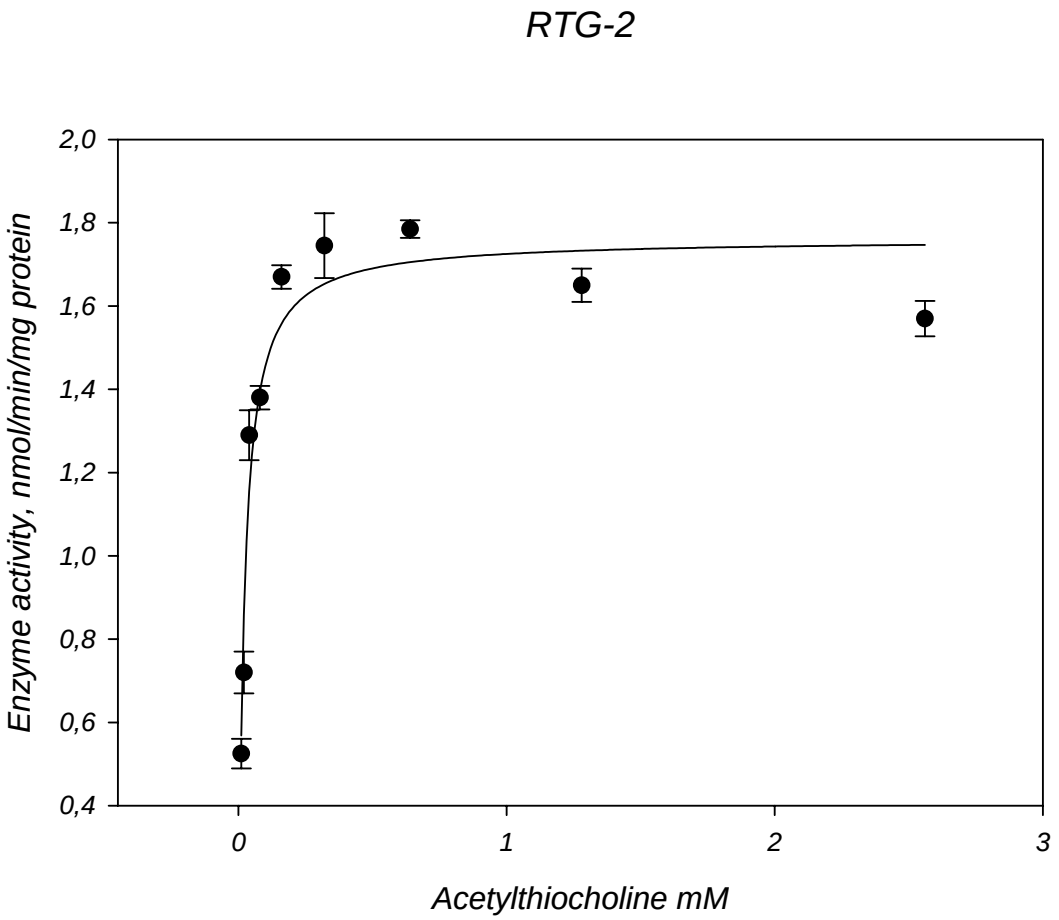

Figure 1. Effect of substrate concentration on esterase activity with acetylthiocholine as substrate in RTG-2 cell line (nmol/min/mg protein +/- SD). The Michaelis-Menten constant (Km) was 0,0210 mM and Vmax was 1,7613 mM

ANNEX 2

2.1. CYTOTOXICITY AND AChE INHIBITION IN BOVINE GRANULOSA CELLS

TABLE 2. *Percentages of cytotoxicity and AChE inhibition in bovine granulosa cells related to the used pesticide concentrations after 48 h of exposure (each value is the mean of 6 replicates for cytotoxicity ans two for AChE determination and three experiments).*

CHLORPYRIFOS

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,1	*0,0*	*0,0*	*0,0*	*0,0*	*2,050*
0,2	*0,0*	*0,0*	*0,0*	*0,0*	*15,88*
0,45	*0,0*	*0,0*	*0,0*	*2,3*	*28,38*
1	*0,0*	*9,90*	*1,5*	*4,39*	*26,91*
2,2	*10,4*	*34,11*	*25,2*	*22,41*	*20,00*
4,5	*18,9*	*37,71*	*30,55*	*27,5*	*29,70*
10	*51,4*	*33,56*	*49,58*	*39,42*	*28,52*
45	*62,5*	*48,26*	*52,86*	*49,42*	*31,47*
100	*65,8*	*55,64*	*64,16*	*54,62*	*79,41*
200	*85,6*	*83,20*	*84,8*	*59,89*	*95,58*

CARBOFURAN

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,045	*0,0*	*0,0*	*0,0*	*0,0*	*4,85*
0,1	*0,0*	*0,0*	*0,0*	*0,0*	*12,35*
0,2	*0,0*	*0,0*	*0,0*	*0,0*	*16,91*
1	*5,70*	*10,75*	*2,2*	*6,57*	*21,32*
2,2	*13,75*	*22,62*	*15,95*	*16,73*	*24,26*
4,5	*32,0*	*36,70*	*29,65*	*30,81*	*25,73*
10	*47,80*	*48,45*	*45,8*	*43,71*	*33,82*
45	*53,1*	*49,30*	*50,7*	*46,66*	*36,17*
100	*54,7*	*62,10*	*61,65*	*54,7*	*51,47*

MIXTURE

Conc. (uM)	% Neutral Red	% Kenacid Blue	% ATP	% Cytotoxicity index	(%) AChE inhibition
0,05	*0,00*	*1,00*	*0,4*	*0,5*	*0,0*
0,2	*10,00*	*11,05*	*10,7*	*9,76*	*2,9*
0,45	*20,85*	*21,90*	*24,55*	*20,34*	*11,8*
1	*24,55*	*19,50*	*25,95*	*20,75*	*16,2*
2,2	*31,10*	*22,80*	*35*	*25*	*22,1*
4,5	*38,50*	*30,30*	*38,8*	*32,02*	*25,0*
10	*44,65*	*45,05*	*46,1*	*41,44*	*33,8*
45	*57,00*	*53,65*	*57,5*	*51,08*	*42,6*
100	*67,75*	*64,30*	*69,6*	*71,51*	*55,9*

2.2.Calculation of Michaelis-Menten' s constant for acetylcholinesterase enzime in Bovine granulosa cells

Substrate : Acetylthiocholine

Nonlinear Regression

[Variables]
x = col(1)
y = col(2)
reciprocal_y = if(y=0;"--";1/abs(y))
reciprocal_ysquare = if(y=0;"--";1/y^2)
[Parameters]
Bmax = max(y) "Auto {{previous: 3,15331}}
Kd = if(x50(x;y)>0; x50(x;y); if(mean(x)=0; 1; abs(mean(x)))) "Auto {{previous: 0,0216594}}
[Equation]
*f = Bmax*abs(x)/(Kd + abs(x))*
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
Bmax>0
Kd>0
[Options]
tolerance = 1e-9
stepsize = 1
iterations=200

R = 0,97975656Rsqr = 0,95992292 Adj Rsqr = 0,95419762

Standard Error of Estimate = 0,1728

	Coefficient	Std. Error	t	P
Bmax	**3,1533**	**0,0897**	**35,1685**	**<0,0001**
Kd	**0,0217**	**0,0033**	**6,5490**	**0,0003**

Analysis of Variance:

	DF	SS	MS	F	P
Regression	1	5,0043	5,0043	167,6634	<0,0001
Residual	7	0,2089	0,0298		
Total	8	5,2132	0,6516		

PRESS = 0,3660

Durbin-Watson Statistic = 2,0307

Normality Test: K-S Statistic = 0,1912 Significance Level = 0,8596

Constant Variance Test: Passed (P = 0,6758)

Power of performed test with alpha = 0,0500: 0,9999

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	3,1268	-0,1818	-1,0526	-1,2161	-1,2678
2	3,1008	-0,1708	-0,9888	-1,1299	-1,1569
3	3,0501	0,1499	0,8678	0,9737	0,9695
4	2,9534	0,1066	0,6170	0,6755	0,6468
5	2,7773	0,1527	0,8837	0,9482	0,9403
6	2,4815	-0,0815	-0,4716	-0,5122	-0,4834
7	2,0456	0,2144	1,2408	1,4385	1,5869
8	1,5139	-0,1839	-1,0642	-1,3046	-1,3884
9	0,9960	-0,0560	-0,3242	-0,3792	-0,3548

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	0,2476	0,2508	-0,7336
2	0,1952	0,2342	-0,6397
3	0,1228	0,2058	0,4935
4	0,0453	0,1656	0,2881
5	0,0680	0,1315	0,3659
6	0,0236	0,1524	-0,2050
7	0,3559	0,2560	0,9307
8	0,4280	0,3346	-0,9845
9	0,0265	0,2692	-0,2153

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	3,1268	2,9222	3,3315	2,6700	3,5837
2	3,1008	2,9032	3,2985	2,6470	3,5547
3	3,0501	2,8648	3,2354	2,6015	3,4987
4	2,9534	2,7872	3,1196	2,5124	3,3944
5	2,7773	2,6292	2,9255	2,3428	3,2119
6	2,4815	2,3220	2,6410	2,0429	2,9200
7	2,0456	1,8390	2,2523	1,5878	2,5035
8	1,5139	1,2775	1,7502	1,0419	1,9858
9	0,9960	0,7841	1,2080	0,5358	1,4562

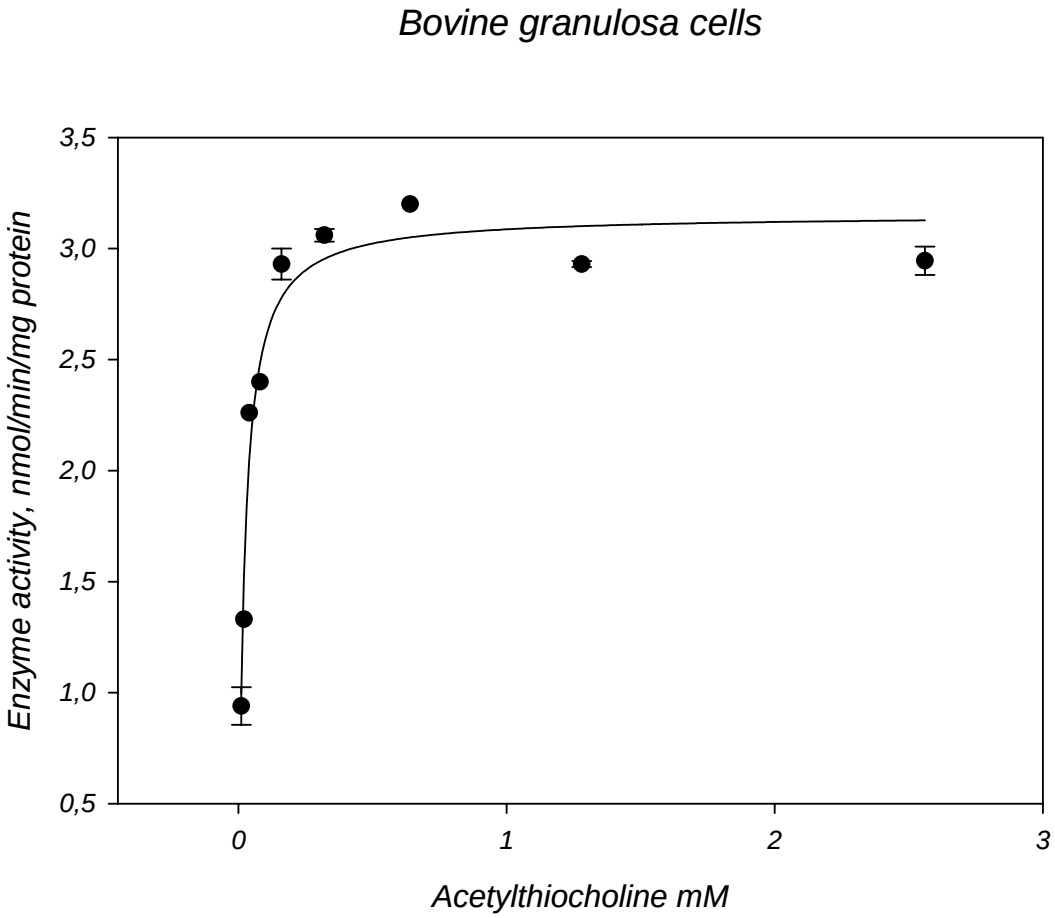

Figure 2. . Effect of substrate concentration on esterase activity with acetylthiocholine as substrate in RTG-2 cell line (nmol/min/mg protein +/- SD). The Michaelis-Menten constant (Km) was 0,0217 mM and Vmax was 3,1533 mM

ANNEX 3

3.1.ACUTE TOXICITY IN *Danio rerio*

TABLE 3. *Percentages of mortality and AChE inhibition in Danio rerio related to the used pesticide concentrations after 96 h of exposure (each value is the mean of 5 fishes and two experiments).*

CHLORPYRIFOS

Conc. (uM)	%mortality	(%) AChE head	(%) AChE muscle	(%) AChE liver
0,014	*0*	*0*	*0*	*0*
0,1	*0*	*5,35*	*0*	*0,8*
0,24	*0*	*72,2*	*59,3*	*41,7*
4,5	*0*	*75,1*	*66,3*	*64,1*
45	*20*	*84,3*	*76,8*	*75,3*
100	*57*	*91,8*	*92,4*	*88,1*
200	*100*	*97,2*	*96,8*	*91,45*

CARBOFURAN

Conc. (uM)	%mortality	(%) AChE head	(%) AChE muscle	(%) AChE liver
0,1	*0*	*0*	*0*	*0*
4,5	*0*	*0*	*0*	*0*
21	*20*	*23,1*	*4*	*0*
45	*80*	*49,4*	*53,9*	*0*
100	*100*	-	-	-

MIXTURE

Conc. (uM)	%mortality	(%) AChE head	(%) AChE muscle	(%) AChE liver
0,01	*0*	*0*	*0*	*0*
0,045	*0*	*0*	*0*	*0*
0,1	*0*	*0*	*0*	*0*
0,45	*0*	*0*	*0*	*0*
4,5	*0*	*14,5*	*0*	*9,2*
21	*20*	*28,5*	*0*	*16,0*
45	*90*	*83,3*	*0*	*74,1*

3.2.Calculation of Michaelis-Menten' s constant for acetylcholinesterase enzime in head zebra fish. (*Danio rerio*)

Substrate : Acetylthiocholine

Nonlinear Regression

[Variables]
x = col(9)
y = col(10)
reciprocal_y = if(y=0;"--";1/abs(y))
reciprocal_ysquare = if(y=0;"--";1/y^2)
[Parameters]
Bmax = max(y) "Auto {{previous: 178,054}}
Kd = if(x50(x;y)>0; x50(x;y); if(mean(x)=0; 1; abs(mean(x)))) "Auto {{previous: 0,198241}}
[Equation]
*f = Bmax*abs(x)/(Kd + abs(x))*
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
Bmax>0
Kd>0
[Options]
tolerance = 1e-9
stepsize = 1
iterations=200

R = 0,98098398Rsqr = 0,96232957 Adj Rsqr = 0,95291196

Standard Error of Estimate = 15,9075

	Coefficient	Std. Error	t	P
Bmax	**178,0537**	**14,8418**	**11,9968**	**0,0003**
Kd	**0,1982**	**0,0696**	**2,8503**	**0,0464**

Analysis of Variance:

	DF	SS	MS	F	P
Regression	*1*	*25857,4771*	*25857,4771*	*102,1841*	*0,0005*
Residual	*4*	*1012,1920*	*253,0480*		
Total	*5*	*26869,6691*	*5373,9338*		

PRESS = 4573,0992

Durbin-Watson Statistic = 2,4652

Normality Test: K-S Statistic = 0,3281 Significance Level = 0,4537

Constant Variance Test: Passed (P = 0,0600)

Power of performed test with alpha = 0,0500: 0,9805

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	*8,5504*	*-0,3804*	*-0,0239*	*-0,0242*	*-0,0210*
2	*16,3172*	*-3,5721*	*-0,2246*	*-0,2336*	*-0,2037*
3	*51,1941*	*-9,0372*	*-0,5681*	*-0,7487*	*-0,6993*

4	*109,9434*	*0,1873*	*0,0118*	*0,0161*	*0,0139*
5	*154,1756*	*23,9290*	*1,5043*	*1,8767*	*4,7010*
6	*171,5149*	*-18,5738*	*-1,1676*	*-1,9923*	*-19,6888*

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	*0,0000*	*0,0232*	*-0,0032*
2	*0,0022*	*0,0760*	*-0,0584*
3	*0,2066*	*0,4243*	*-0,6003*
4	*0,0001*	*0,4624*	*0,0129*
5	*0,9798*	*0,3575*	*3,5067*
6	*3,7936*	*0,6565*	*-27,2211*

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	*8,5504*	*1,8166*	*15,2841*	*-36,1263*	*53,2270*
2	*16,3172*	*4,1429*	*28,4914*	*-29,4963*	*62,1306*
3	*51,1941*	*22,4248*	*79,9633*	*-1,5158*	*103,9039*
4	*109,9434*	*79,9093*	*139,9775*	*56,5326*	*163,3541*
5	*154,1756*	*127,7678*	*180,5833*	*102,7166*	*205,6346*
6	*171,5149*	*135,7285*	*207,3014*	*114,6702*	*228,3597*

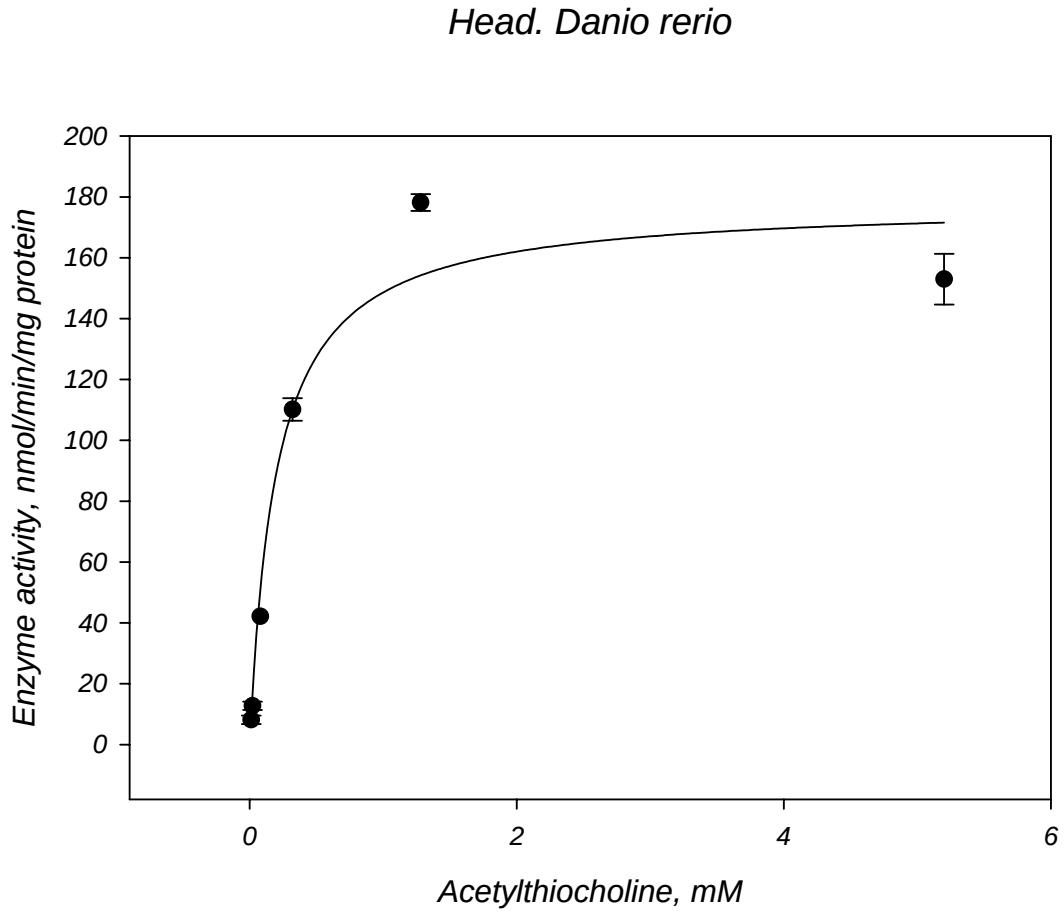

Figure 3 . *Effect of substrate concentration on esterase activity with acetylthiocholine as substrate in head of Danio rerio (nmol/min/mg protein +/- SD). The Michaelis-Menten constant (Km) was 0,1982 mM and Vmax was 178,05 mM*

3.3.Calculation of Michaelis-Menten' s constant for acetylcholinesterase enzime in muscle zebra fish. (*Danio rerio*)

Substrate : Acetylthiocholine

Nonlinear Regression

```
[Variables]
x = col(1)
y = col(2)
reciprocal_y = if(y=0;"--";1/abs(y))
reciprocal_ysquare = if(y=0;"--";1/y^2)
[Parameters]
Bmax = max(y) "Auto {{previous: 577,925}}
Kd = if(x50(x;y)>0; x50(x;y); if(mean(x)=0; 1; abs(mean(x))))  "Auto {{previous: 0,211364}}
[Equation]
f = Bmax*abs(x)/(Kd + abs(x))
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
Bmax>0
Kd>0
[Options]
tolerance = 1e-9
stepsize = 1
iterations=200
```

R = 0,97989371Rsqr = 0,96019169 Adj Rsqr = 0,95023961

Standard Error of Estimate = 53,6009

	Coefficient	Std. Error	t	P
Bmax	**577,9249**	**50,9086**	**11,3522**	**0,0003**
Kd	**0,2114**	**0,0776**	**2,7239**	**0,0528**

Analysis of Variance:

	DF	SS	MS	F	P
Regression	*1*	*277197,1756*	*277197,1756*	*96,4815*	*0,0006*
Residual	*4*	*11492,2381*	*2873,0595*		
Total	*5*	*288689,4137*	*57737,8827*		

PRESS = 52971,9621

Durbin-Watson Statistic = 2,4421

Normality Test: K-S Statistic = 0,3451 Significance Level = 0,3897

Constant Variance Test: Passed (P = 0,0600)

Power of performed test with alpha = 0,0500: 0,9780

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	*26,1075*	*-6,4997*	*-0,1213*	*-0,1225*	*-0,1063*
2	*49,9582*	*-17,7453*	*-0,3311*	*-0,3431*	*-0,3016*

3	158,6814	-27,7290	-0,5173	-0,6715	-0,6173
4	348,0404	-1,4017	-0,0262	-0,0362	-0,0314
5	496,0185	81,0123	1,5114	1,8832	4,8430
6	555,3516	-61,6542	-1,1502	-1,9973	-33,2567

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	0,0002	0,0209	-0,0155
2	0,0044	0,0691	-0,0822
3	0,1544	0,4064	-0,5108
4	0,0006	0,4794	-0,0301
5	0,9797	0,3559	3,5998
6	4,0193	0,6683	-47,2094

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	26,1075	4,5930	47,6219	-124,2596	176,4746
2	49,9582	10,8383	89,0780	-103,9177	203,8340
3	158,6814	63,8070	253,5559	-17,8081	335,1710
4	348,0404	244,9995	451,0812	167,0298	529,0509
5	496,0185	407,2396	584,7974	322,7294	669,3075
6	555,3516	433,6884	677,0148	363,1295	747,5737

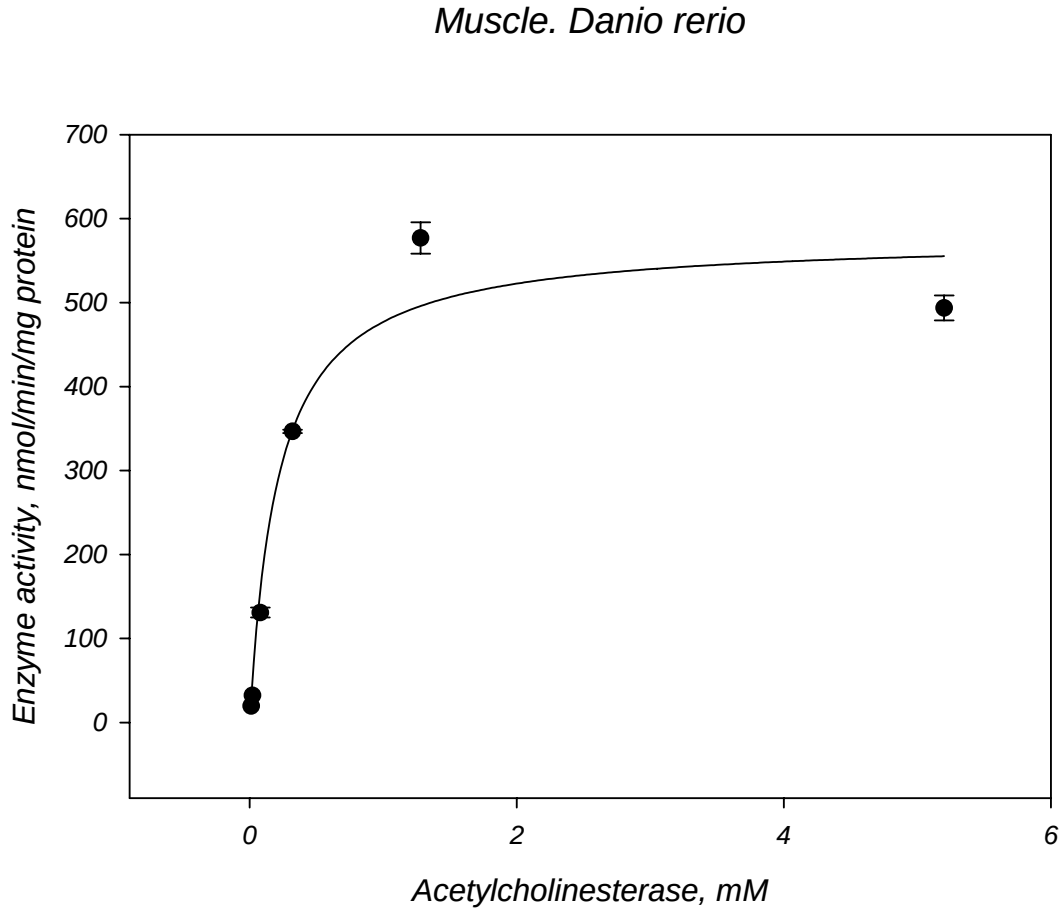

Figure 4 . Effect of substrate concentration on esterase activity with acetylthiocholine as substrate in muscle of Danio rerio (nmol/min/mg protein +/- SD). The Michaelis-Menten constant (Km) was 0,2114 mM and Vmax was 577,92 mM

3.4.Calculation of Michaelis-Menten' s constant for acetylcholinesterase enzime in liver zebra fish. (*Danio rerio*)

Substrate : Acetylthiocholine

Nonlinear Regression

[Variables]
x = col(1)
y = col(2)
reciprocal_y = if(y=0;"--";1/abs(y))
reciprocal_ysquare = if(y=0;"--";1/y^2)
[Parameters]
Bmax = max(y) "Auto {{previous: 18,4893}}
Kd = if(x50(x;y)>0; x50(x;y); if(mean(x)=0; 1; abs(mean(x)))) "Auto {{previous: 0,165111}}
[Equation]
*f = Bmax*abs(x)/(Kd + abs(x))*
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare
[Constraints]
Bmax>0
Kd>0
[Options]
tolerance = 1e-9
stepsize = 1
iterations=200

R = 0,98087950Rsqr = 0,96212459 Adj Rsqr = 0,95265573

Standard Error of Estimate = 1,6375

	Coefficient	Std. Error	t	P
Bmax	**18,4893**	**1,4553**	**12,7047**	**0,0002**
Kd	**0,1651**	**0,0562**	**2,9391**	**0,0424**

Analysis of Variance:

	DF	SS	MS	F	P
Regression	*1*	*272,4499*	*272,4499*	*101,6094*	*0,0005*
Residual	*4*	*10,7254*	*2,6813*		
Total	*5*	*283,1752*	*56,6350*		

PRESS = 44,2464

Durbin-Watson Statistic = 2,5504

Normality Test: K-S Statistic = 0,2760 Significance Level = 0,6749

Constant Variance Test: Passed (P = 0,0600)

Power of performed test with alpha = 0,0500: 0,9802

Regression Diagnostics:

Row	Predicted	Residual	Std. Res.	Stud. Res.	Stud. Del. Res.
1	*1,0559*	*0,2108*	*0,1287*	*0,1308*	*0,1135*
2	*1,9976*	*-0,4143*	*-0,2530*	*-0,2665*	*-0,2329*

3	*6,0346*	*-0,6513*	*-0,3977*	*-0,5467*	*-0,4922*
4	*12,1963*	*-0,0998*	*-0,0609*	*-0,0795*	*-0,0689*
5	*16,3768*	*2,4964*	*1,5245*	*1,9095*	*5,5611*
6	*17,9203*	*-1,9604*	*-1,1972*	*-1,9510*	*-7,6824*

Influence Diagnostics:

Row	Cook'sDist	Leverage	DFFITS
1	*0,0003*	*0,0314*	*0,0204*
2	*0,0039*	*0,0988*	*-0,0771*
3	*0,1329*	*0,4707*	*-0,4641*
4	*0,0022*	*0,4131*	*-0,0578*
5	*1,0371*	*0,3626*	*4,1943*
6	*3,1514*	*0,6235*	*-9,8855*

95% Confidence:

Row	Predicted	Regr. 5%	Regr. 95%	Pop. 5%	Pop. 95%
1	*1,0559*	*0,2508*	*1,8609*	*-3,5612*	*5,6730*
2	*1,9976*	*0,5683*	*3,4270*	*-2,7681*	*6,7634*
3	*6,0346*	*2,9156*	*9,1536*	*0,5212*	*11,5480*
4	*12,1963*	*9,2743*	*15,1184*	*6,7919*	*17,6008*
5	*16,3768*	*13,6392*	*19,1144*	*11,0698*	*21,6838*
6	*17,9203*	*14,3305*	*21,5101*	*12,1275*	*23,7130*

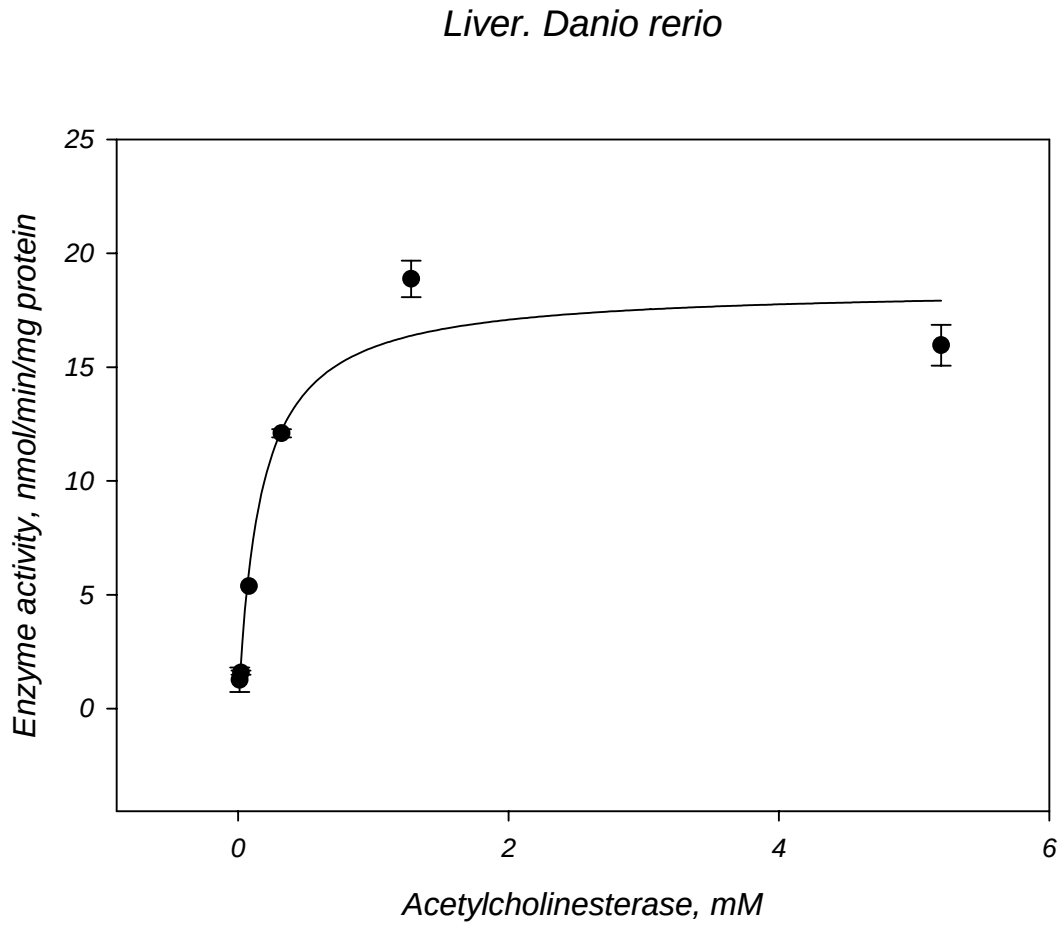

Figure 5. Effect of substrate concentration on esterase activity with acetylthiocholine as substrate in liver of Danio rerio (nmol/min/mg protein +/- SD). The Michaelis-Menten constant (Km) was 0,1651 mM and Vmax was 18,489 mM

ANNEX 4

4.1.ACUTE TOXICITY IN MICROALGAE

TABLE 4. *Percentages of growth inhibition in Chlorella vulgaris, Selenastrum capricornutum, Scenedesmus subspicatus related to the used pesticide concentrations after 72 h of exposure (n_1- n_6: repetitions).*

CHLORPYRIFOS

a) *Selenastrum capricornutum*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
25	*10,20*	*11,50*	*13,04*	*14,29*	*12,50*	*10,40*
50	*19,57*	*21,74*	*24,50*	*23,21*	*22,60*	*25,40*
100	*34,78*	*37,80*	*38,70*	*39,29*	*35,60*	*37,40*
200	*67,39*	*65,22*	*63,04*	*66,07*	*62,50*	*67,80*

b) *Chorella vulgaris*

Conc. (uM))	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
25	*1,32*	*0,90*	*0,66*	*1,92*	*0,64*	*1,28*
50	*33,55*	*27,40*	*29,20*	*33,33*	*31,10*	*30,50*
100	*44,08*	*43,40*	*46,71*	*41,03*	*45,51*	*44,23*
200	*59,21*	*57,89*	*58,55*	*58,33*	*62,18*	*66,03*

c) *Scenedesmus subspicatus*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
25	*11,90*	*16,67*	*14,29*	*21,95*	*14,63*	*12,20*
50	*40,48*	*33,33*	*35,71*	*39,02*	*31,71*	*34,15*
100	*59,52*	*61,90*	*57,14*	*53,66*	*48,78*	*63,41*
200	*95,24*	*92,86*	*95,24*	*82,93*	*90,24*	*87,80*

CARBOFURAN

a) *Selenastrum capricornutum*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
3,7	*9,16*	*8,96*	*8,96*	*10,10*	*8,57*	*9,20*
13,5	*19,40*	*21,40*	*25,35*	*26,40*	*19,50*	*18,57*
27,1	*35,20*	*36,40*	*35,40*	*38,70*	*35,40*	*37,50*
58,7	*51,40*	*49,80*	*42,50*	*41,50*	*48,60*	*51,30*
131,0	*58,60*	*58,70*	*62,40*	*68,57*	*61,40*	*57,90*
185,3	*75,52*	*72,10*	*70,10*	*75,60*	*69,50*	*74,50*

b) *Chorella vulgaris*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
3,7	*7,46*	*8,96*	*8,96*	*5,71*	*8,57*	*10,00*
13,5	*22,39*	*23,88*	*25,37*	*25,71*	*27,14*	*28,57*
27,1	*55,22*	*56,72*	*55,22*	*48,57*	*55,71*	*57,14*
58,7	*61,19*	*59,70*	*62,69*	*71,43*	*78,57*	*71,43*
131,0	*88,06*	*98,51*	*82,09*	*78,57*	*90,00*	*87,14*
185,3	*95,52*	*98,51*	*94,03*	*95,71*	*100*	*94,29*
262,1	*100*	*100*	*100*	*110*	*120*	*100*

c) *Scenedesmus subspicatus*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
3,7	*4,50*	*5,90*	*6,10*	*4,80*	*5,70*	*5,90*
13,5	*15,40*	*18,60*	*17,40*	*19,80*	*16,90*	*18,90*
27,1	*30,40*	*32,60*	*35,40*	*28,70*	*34,90*	*33,60*
58,7	*48,90*	*44,70*	*43,60*	*45,38*	*47,90*	*50,10*
131,0	*70,10*	*64,80*	*72,50*	*62,30*	*67,50*	*66,40*
185,3	*88,90*	*79,40*	*79,50*	*81,50*	*88,40*	*85,60*

MIXTURE

a) *Selenastrum capricornutum*

Conc. (uM))	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
6,0	*2,31*	*3,10*	*3,20*	*2,20*	*2,50*	*3,10*
12,5	*26,40*	*27,70*	*22,60*	*25,40*	*27,40*	*19,10*
25,0	*37,80*	*39,60*	*36,50*	*38,40*	*37,80*	*35,70*
50,0	*52,50*	*53,50*	*56,40*	*59,40*	*55,40*	*53,60*
100,0	*61,40*	*68,10*	*67,20*	*63,40*	*65,60*	*60,40*

b) *Chorella vulgaris*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
6,0	*23,10*	*22,00*	*21,50*	*22,60*	*26,50*	*33,10*
12,5	*36,40*	*37,80*	*32,50*	*35,40*	*37,80*	*39,10*
25,0	*47,80*	*49,30*	*46,50*	*44,70*	*45,80*	*45,70*
50,0	*62,50*	*63,50*	*66,50*	*59,40*	*60,10*	*62,50*
100,0	*81,40*	*80,50*	*77,98*	*79,40*	*75,60*	*80,50*

c) *Scenedesmus subspicatus*

Conc. (uM)	n_1 (%)	n_2 (%)	n_3 (%)	n_4 (%)	n_5 (%)	n_6 (%)
6,0	*6,50*	*5,50*	*8,40*	*7,50*	*7,70*	*6,90*
12,5	*18,20*	*19,50*	*15,60*	*17,90*	*19,40*	*15,60*
25,0	*33,60*	*34,50*	*39,50*	*32,50*	*33,30*	*36,39*
50,0	*49,60*	*55,20*	*49,80*	*50,20*	*47,80*	*46,50*
100,0	*63,20*	*65,50*	*66,90*	*65,80*	*60,20*	*69,20*

ANNEX 5

5.1.ACUTE TOXICITY IN *Vibrio fischeri*

TABLE 5. *Relation of values of emited light reduction (GAMMA, Γ) in Vibrio fischeri related to pesticide concentrations after 5 and 15 min (n_1- n_3: repetitions).*

CHLORPYRIFOS

	5 minutes			15 minutes		
Conc. (uM)	Γ n_1	Γ n_2	Γ n_3	Γ n_1	Γ n_2	Γ n_3
50	*0,023*	*0,032*	*0,031*	*0,220*	*0,167*	*0,109*
100	*0,076*	*0,080*	*0,100*	*0,334*	*0,277*	*0,261*
200	*0,835*	*0,681*	*0,833*	*1,348*	*1,340*	*1,911*
400	*7,507*	*7,661*	*8,080*	*10,446*	*10,164*	*9,388*

CARBOFURAN

	5 minutes			15 minutes		
Conc. (uM)	Γ n_1	Γ n_2	Γ n_3	Γ n_1	Γ n_2	Γ n_3
12,5	*0,299*	*0,253*	*0,254*	*0,350*	*0,259*	*0,272*
25	*0,374*	*0,388*	*0,458*	*0,482*	*0,411*	*0,473*
50	*0,666*	*0,618*	*0,816*	*0,928*	*0,639*	*0,819*
100	*1,837*	*1,171*	*1,397*	*1,860*	*1,186*	*1,560*
200	*3,917*	*2,600*	*3,780*	*4,784*	*3,642*	*4,355*

MIXTURE

	5 minutes			15 minutes		
Conc. (uM)	Γ n_1	Γ n_2	Γ n_3	Γ n_1	Γ n_2	Γ n_3
25	*0,481*	*0,188*	*0,333*	*0,556*	*0,259*	*0,327*
50	*0,753*	*0,574*	*0,639*	*0,950*	*0,717*	*0,711*
100	*1,536*	*1,063*	*1,309*	*2,038*	*1,394*	*1,175*
200	*3,720*	*2,936*	*3,203*	*5,300*	*4,973*	*4,558*

TABLE 6.- *Relation of values of percentage of inhibition of bioluminiscence in Vibrio fischeri related to pesticide concentrations (n_1- n_3: repetitions).*

CHLORPYRIFOS

	5 minutes			15 minutes		
Conc. (uM)	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**
50	*2,27*	*3,1*	*3,01*	*1,22*	*0,76*	*0,48*
100	*7,07*	*7,39*	*9,13*	*9,69*	*9,32*	*12,45*
200	*45,51*	*40,51*	*45,43*	*48,49*	*50,52*	*62,09*
400	*88,24*	*88,45*	*88,98*	*89,48*	*89,62*	*89,37*

CARBOFURAN

	5 minutes			15 minutes		
Conc. (uM)	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**
12,5	*23,04*	*20,21*	*20,28*	*25,94*	*20,59*	*21,36*
25	*27,21*	*27,96*	*31,43*	*32,50*	*29,12*	*32,09*
50	*39,96*	*38,20*	*44,92*	*48,13*	*39,00*	*45,03*
100	*64,75*	*53,93*	*58,29*	*65,03*	*54,25*	*60,94*
200	*79,66*	*72,22*	*79,08*	*82,71*	*78,46*	*81,33*

MIXTURE

	5 minutes			15 minutes		
Conc. (uM)	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**	**%INH**
25	*32,46*	*15,82*	*24,98*	*35,41*	*17,06*	*24,66*
50	*42,96*	*36,45*	*38,97*	*48,72*	*41,74*	*41,54*
100	*60,57*	*51,52*	*56,69*	*67,09*	*58,23*	*54,03*
200	*78,81*	*74,59*	*76,21*	*84,13*	*83,26*	*82,01*

ANNEX 6"

TABLE 7. *The obtained toxicity units (TU) for each pesticide single and in mixture and their toxicologic interactions.*

Test species	Endpoint and exposure time	TU chlorpyrifos	TU carbofuran	TU chlorpyrifos+ TU carbofuran		Toxicology interactions
In vivo						
Bacteria						
***Vibrio fischeri*"**	**% Bioluminiscence inhibition 5min**	*0,166*	*0,555*	*0,721*	*<1*	*Synergistic*
	% Bioluminiscence inhibition 15min	*0,159*	*0,555*	*0,715*	*<1*	*Synergistic*
Algae						
Chlorella vulgaris	**% Growth inhibition 72h**	*0,098*	*0,461*	*0,559*	*<1*	*Synergistic*
Selenastrum capricornutum	**% Growth inhibition 72h**	*0,175*	*0,367*	*0,542*	*<1*	*Synergistic*
Scenedesmus subspicatus	**% Growth inhibition 72h**	*0,360*	*0,452*	*0,812*	*<1*	*Synergistic*
Fish						
Danio rerio	**%mortality 96h**	*0,223*	*0,562*	*0,785*	*<1*	*Synergistic*
	%AChE head inhibition 96h	*14,02*	*0,262*	*14,28*	*>1*	*Antagonistic*
	%AChE muscle inhibition 96h	*27,78*	*0,472*	*28,24*	*>1*	*Antagonistic*
	%AChE liver inhibition 96h	*5,99*	*0,282*	*6,27*	*>1*	*Antagonistic*
In vitro						
Bovine granulosa cells	**% cytotoxicity 48h**	*0,294*	*0,462*	*0,757*	*<1*	*Synergistic*
	%AChE inhibition 48h	*1,871*	*0,506*	*2,377*	*>1*	*Antagonistic*
***RTG-2*"**	**% cytotoxicity 48h**	*0,295*	*0,548*	*0,844*	*<1*	*Synergistic*
	%AChE inhibition 48h	*15,58*	*0,524*	*16,11*	*>1*	*Antagonistic*

ANNEX-OF RELATED COMMUNICATIONS AND PUBLICATIONS

Alañón, P., Riva,M.C., Barata,C, Stacchezzini,S. 2005 Evaluation of the toxicant effects of two neurotoxics in two different cell lines; RTG-2 from fish gonad and granulosa cells from bovine ovary. Congreso Nacional de Toxicologia. Cáceres, Spain. Revista de Toxicología 22(2), 137-138

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Alañón, P., Gibert, C., Riva, M.C. 2002. Toxicity of tannery wastewaters using the luminescent bacteria bioassay, Microtox™. Toxicology letters 135, S1. S95

Alañón P., Riva M.C., Llorente M.T., Castaño A. 2001. Acute toxicity of residual baths from leather tanning industry on RTG-2 cytotoxicity test. Proceedings of the ETCS 43rd International Meeting, 111. Granada. Spain.

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Riva, M.C.; Alañón, P.; Castaño, A. 2005. Cytotoxicity of Leather Processing Effluents on RTG-2 Fish Cell Line. Bulletin of environmental contamination and toxicology 75 (1), 34-41

Riva, M.C.; Gibert, C.; Alañón, P.; Cornellas, A. 2002. Toxicity of tannery wastewaters using two bioassays.Toxicology letters 135 S1. S96.

Ribó, J.M.; Riva, M.C.; Alañón, P. 2005. 18th Annual meeting of SETAC Europe. Variability of results for mixture toxicity with the luminiscent bacteria bioassay. Lille, France.

Stacchezzini, S.; Alañón, P.; Riva, M.C. and Caloni, F. 2006. Preliminary Evaluation of Carbofuran cytotoxicity and steroidogenesis effects on bovine granulosa cells " 46th ETCS International Meeting, 3rd International Joint Meeting AICC-CELLTOX "In vitro cytotoxicity mechanisms" Verona, Italy.