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Use of *Caenorhabditis elegans* based assays as an alternative strategy in toxicity testing

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To my family and friends, to my tutors Isaias Salvador Sanmartín Santos and Dr. Nico van den Brink, to the head of the department of toxicology of the Wageningen University & research, Dr. Ivonne Rietjens, and my colleagues Isaac Omwenga y Shensheng Zhao.

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Abbreviation list

C. elegans - *Caenorhabditis elegans*

E. coli - *Escherichia coli*

AChE - Acetylcholinesterase enzyme

OP - Organophosphate

QSAR - Quantitative structure-activity relationship

VNC - Ventral nerve cord

DNC - Dorsal nerve cord

KO - Knockout

CGC - Caenorhabditis Genetics Center

COPAS - Complex Object Parametric Analyzer and Sorter

CPF - Chlorpyrifos

PFF - Profenofos

NGM - Nematode Growing Medium

ATC - Acetylcholine

DTNB - Ellman’s reagent

RESUMEN/ ABSTRACT

Use of Caenorhabditis elegans based assays as an alternative strategy in toxicity testing

RESUMEN

Los modelos *in vivo* son una herramienta de trabajo esencial en el laboratorio de biomedicina. En particular son imprescindibles para obtener respuestas biológicas completas a ciertos estímulos o sustancias, que deben evaluarse en el contexto de un organismo completo. La toxicología en particular es un campo que demanda continuamente de nuevos modelos, los cuales deben proporcionar a los investigadores resultados confiables en un corto periodo de tiempo.

El uso en investigación del nematodo *Caenorhabditis elegans* (*C. elegans*) fue promovido y desarrollado por Sydney Brenner. Es un invertebrado pequeño (gusano redondo) utilizado extensamente en la investigación biológica básica, que está alcanzando una gran popularidad como modelo *in vivo*. Su genoma está extensamente anotado, y muestra una inesperada homología de muchos de sus genes con los genes humanos, constituyendo por ello un modelo muy apropiado por su facilidad de cultivo y manejo.

En el presente trabajo, se desarrolladó un test de evaluación de la toxicidad de pesticidas organofosforados en el modelo de *C. elegans*.

Se han testado dos tipos de organofosfatos presentes en pesticidas, clorpirifós-oxon y profenofos, tanto *in vivo* (*C. elegans*) como *in vitro* (ensayos enzimáticos con acetilcolinesterasa recombinante humana), comparándose los valores de IC_{50} y EC_{50} obtenidos.

Para el test *in vivo* se utilizó el WMicrotraker One, instrumento que permite medir los efectos del tratamiento aplicado sobre la locomoción del nematodo y otros parámetros.

Palabras clave: *C. elegans*, *in vivo*, AChE, clorpirifós, profenofos, WMicrotracker One, espectrofotómetro, DNTB

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ABSTRACT

In vivo models are an essential working tool in the biomedicine laboratory. Specially, they are important for obtaining complete biological responses to certain stimuli or substances, which needs to be evaluated as a whole complete organism. Toxicology particularly is a demanding field of new models, which needs to administer investigators, reliable results in a short period of time.

The use in investigations of the nematode *Caenorhabditis elegans (C. elegans)* was first boosted and developed by Sydney Brenner. It is a small invertebrate animal (round worm), used extensively in basic biological investigations, and reaching a great popularity as an *in vivo* model. Their genome is already annotated, and shows an unexpected range of homology between their genes and the human genome, consequently, constituting a very appropriate model by their facility of cultivation and management inside the lab.

At the present work, it was developed an evaluation toxicity test of organophosphate pesticides on the model *C. elegans*.

Two kinds of organophosphates present in pesticides were evaluated, chlorpyrifos-oxon and profenofos,for both *in vivo* (*C. elegans*) and *in vitro* (enzymatic assays with the human recombinant protein), sharing the EC_{50} and IC_{50} obtained values.

For the *in vivo* test, the WMicrotracker One was used , a machine which allows reading of the effects of a treatment applied on the nematode's locomotion and other parameters.

Key words: *C. elegans*, *in vivo*, AChE, chloropyrifos, profenofos, WMicrotracker One, spectrophotometer, DNTB

Use of Caenorhabditis elegans based assays as an alternative strategy in toxicity testing

INTRODUCTION

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INTRODUCTION

Producing new *in vivo* models for toxicity testing has been an important issue over the last years, specially doe to the need for a reduction on the use of mammalian test animals. Making scientists to search for alternatives, which enters inside the parameters of an acceptable laboratory assessment model, in terms of costs, maintenance, and potential similarities with possible human outcomes (Hunt, 2017). This risk assessment tests are mostly performed, in order to obtain a collection of reliable data, which could be extrapolated to a human population within an ambient exposure (Bhattacharya et al., 2011). Advancing the researcher with a variety of possible non-expected effects. Therefore, the use and standardization of a diversity of animals and testing methods, makes it easier to evaluate the risks of new chemicals and their effects, in a whole organism.

Risk assessment strategies

Being an expansive area, different kind of approaches can be used to assess toxicity tests including, *in vitro*, *in vivo* (in which animal models are being stablished) and the computational *in silico* testing strategy (Settivari et al., 2015). **Table 1** shows some differences and similarities between these tree methods, as their strengths and weaknesses in each category. As well, a broad variety of endpoints can be measured within each technique. For *in vitro* techniques some examples include: eye irritation, skin corrosion, developmental toxicity, endocrine toxicity, etc (Settivari et al., 2015). Within the *in vivo* pathway, the 'six-pack' acute toxicity testing is classically used by researchers, including a number of different measurable endpoints from the whole organism: oral, dermal, inhalation, eye irritation and skin irritation/sensibilization toxicity assays (Prior et al., 2019).

Table 1. Comparative endpoints of available toxicity testing approaches (Settivari et al., 2015).

	Predictive toxicology approaches		
	In silico approach	In vitro biologic profiling	Conventional testing approaches
Test systems	Computational	Predominantly cell- and molecular-based	Animal-based testing
Animal usage	Not applicable	None to minimal	Extensive
Expense (per chemical)	$	$$	$$$$
Study duration	Days	Days to weeks	Months to years
Test material requirement	None	Micrograms to milligrams	Grams to kilograms
Throughput	Moderate to high	Moderate to high	Low
Dose levels	Not applicable[a]	Many (5 to 10) dose levels	Typically 3

[a]Exceptions include physiologically based pharmacokinetic modeling and virtual tissue models

On the other hand, to first stablish a novel technique as a new alternative toxicity plan, a series of characteristics and quality, needs to be accomplish. This is essential, to be able to guarantee a correlation between the result given by the technique and reliable significant data (Hunt, 2017). Specially, with the *in vitro* and *in silico* methods, in which data is obtained, but a biological response is not performed and possible human responses cannot be fully predicted.

Starting with the *in vitro* practices, a 2- or 3D cell cultures profile is designed with the desired morphology and biochemistry. This arrangement depends on the aimed assay and the possible outcoming adverse effects (Settivari et al., 2015). Some of the methods used, are for instance, primary human cells for risk assessments. This modus has a higher potential accuracy in terms of human-specific metabolism and methods of action than *in vivo* animal models, overcoming limitations of cross-species predictions (Scott et al., 2013). Further, the use of immortalized cell lines is another assessment strategy, which generates an unlimited number of cloned cells ready for testing in a short period of time (Allen et al., 2005). However, proceeding with this method, a big amount of false positives appears (Knight et al., 2009). This types of cells, also loose viability and stability overtime; i.e., in genotoxic assessments of cosmetic ingredients, which usually test with immortalized cells as an animal alternative method, currently is investigating alternative methods to reduce the appearance of false negatives (Pfuhler et al., 2010). This results, in an incomplete and not complex organismal level response prediction for *in vitro* assays (Hunt, 2017).

Secondly, in predictive toxicology, various methods are available. For example, cheminformatics techniques as an *in silico* approach. It consists of computational analyses using mathematical, chemical, and statistical methods, to predict human outcomes (Settivari et al., 2015). This tool can easily analyze new chemicals giving relevant information about the compound and its analogs. Using the structure-based (quantitative structure-activity relationship [QSAR]) predictive approach, a relationship between structure and human conditions, can be predicted, giving a first insight about how the compound can biologically disrupt metabolic pathways (Settivari et al., 2015). Even though, many efforts have been put to develop this computational method, prediction errors occur, i.e. failure on the use of endpoint units, not taking into account data in an heterogenety manner, errors in the descriptor values and another large amount of omissions of important data points (Cherkasov et al., 2014). This means that data obtained with this *in silico* approach, have low reliability compared with other methods.

Additionally, mammalian animal models, the classical model for laboratory assessments, opened the door of toxicity testing on organisms which had greater genetic semblances with the human specie, positioning this technique and use of mammalians in laboratories as the first option when preparing an experimental toxicity plan. Their many similarities with humans, including different functional organs and their capacity of mimicking the developmental pathways, as well as obtaining results as a whole organismal level, have placed this method as the 'gold standard' testing technique in the field of toxicology (Hunt, 2017). However, the use of the mammalian models inside the laboratory is becoming obsolete and it is even more confronted with scientific, economic and ethical issues (Settivari et al., 2015). This is giving birth to other methods, which will one day overcome completely the use of mammalian animals in investigations.

The role of nematodes as an in vivo model

The role of nematodes as whole organismal animal models, has increased over the past centuries, starting as, subject of study of many researchers at the end of the 19th century. Nematodes among other aspects, helped to reveal basic aspects of biology including, developmental expression and reproduction (Hamoir, 2003). Most recently, *Caenorhabditis elegans* (*C. elegans*) has become one of the major model system in developmental biology, outstanding on toxicological assays as an alternative for animal testing, launching many companies and research groups inside of this genre of study (Lambshead, 2004; Leung et al., 2008; Hunt, 2017). Reports showed that earlier nematode studies' have already been published, although the main figure to recognize its real potential as a model organism, was seen and reflected on the publication of Brenner's seminal genetics paper (Brenner, 1974), highlighting its research on the nervous system and how it is related with behaviour. Brenner, who was ahead of his time, was one of the first researchers focused on writing standardized procedures for cultivation and management of *C. elegans*. With the advanced techniques of our century, Brenner opened a whole new field of study, inspiring a substantial amount of new researchers into an unexplored territory.

Even though, many organisms from the phylum Nematoda have been studied over many years and their potential as an animal model, *C. elegans* is the choice used worldwide, especially in toxicity assays and testing strategies. Their many similarities with humans is outstanding, so various studies have been performed to characterize this organism

and understand how their biology work (Wormbook), to standardize it as an alternative animal model in toxicity strategies.

The nematode C. elegans

C. elegans, a free-living nematode, reside mainly on soil-type environments in many parts of the world and feed on fungi and bacteria (Hunt, 2017). They are normally grown in agar plates in the laboratory, feed with *Escherichia coli* (*E. coli*) and incubated in the temperature range of 12°C to 25°C (higher temperatures can make the organism become sterile) (Corsi et al., 2015). *C. elegans* can also be cultured in a liquid medium, but this procedure is normally performed for bulk growth of the organism in biochemical studies (Corsi et al., 2015). They can be frozen with liquid nitrogen (−196 °C) in the lab for many years and revive them when it is handy (Stiernagle, 1999). This can prevent the appearance of genetic drifts (Hunt, 2017).

Sexual forms

The wild type *C. elegans*, consists of two sexual forms, hermaphrodite and male form. Hermaphrodites can self-fertilize and produce over 300 self-progeny. Sperm is stored and used later to fertilize the oocytes produced within the gonad of the same animal (Corsi et al., 2015). On the other hand, if a male *C. elegans* mates with an hermaphrodite, they can produce around ~1000 offspring, indicating that for hermaphrodites, the sperm is a limitation factor (Corsi et al., 2015). On an average, the majority of the self-fertilized hermaphrodites produce offsprings which only 0.1-0.2% becomes a male (Corsi et al., 2015). This is due to the rare meiotic non-disjunction found on the X chromosome, which is caused by toxins and stress, occurring rarely in nature (Hunt, 2017). Hermaphrodites are commonly diploid, with five pairs of autosomes and a pair of X chromosomes (XX) (Herman, 2005). Males have the same five pairs of autosomes, but only a single X chromosome (XO) (Herman, 2005). Hermaphrodites are the common form used in testing assays, providing a series of advantages over the use of males. Easy to culture in terms of maintaining stocks of the organism is the main point, specially, a whole population of worms can be produced from one unique nematode and with a low probability of obtaining males (Corsi et al., 2015).

Anatomy

In terms of anatomy, newly hatched *C. elegans* larvae are 0.25 mm long and adults are just a bit over 1mm long (Corsi et al., 2015). They are also formed by transparent tissues, which gives an advantage in terms of easy internal organs study and responses without dissection (Hunt, 2017). As other nematodes, *C. elegans* has a cylindrical, unsegmented body shape, with tapered ends (wormatlas). It is formed of an outer tube and an inner tube, which are separated by the pseudocoelomic space. The most outer tube, is formed by a resistant cuticle, the hypodermis, an excretory system, neurons, and muscles, and the inner tube comprises the pharynx, an intestine, and, in the adult, gonad (wormatlas).

Males and hermaphrodites shows some differences in structure. Males L2 onward start to develop their sexual organs and start to differ from hermaphrodites, being able to differentiate both worms with the help of a microscope (wormatlas). All the body areas of the worm, except the pharynx and the excretory system, shows some differences between both kind of worms (wormatlas). The muscle system, presents 41 extra sex-specific muscles, which hermaphrodites do not present (wormatlas). The nervous system has an additional 91 neurons in males(wormatlas). **Figure 1** shows some structural differences and similarities between the anatomical systems of both hermaphrodites an males. From the nerve ring, the ventral nerve cord (VNC) and the dorsal nerve cord (DNC), run along the whole length of the worm. The pharynx and the intestine can be seen in **Figure 1-B**, as the nervous system and muscles is omitted, to process a better view of the rest of the systems. Anatomical differences regarding the tail form of males and hermaphrodites. A more thin and long tail for hermaphrodites and a fan-like structure in males. In **Figure 1-C**, the cuticle, pseudocoelomic cavity, and the structures in the inner tube can be recognized. The four muscle quadrants is identified in **Figure1-C. Figure 1-A** shows the oocytes and the embryos inside the hermaphrodite's body, including the spermateca (Corsi et al., 2015).

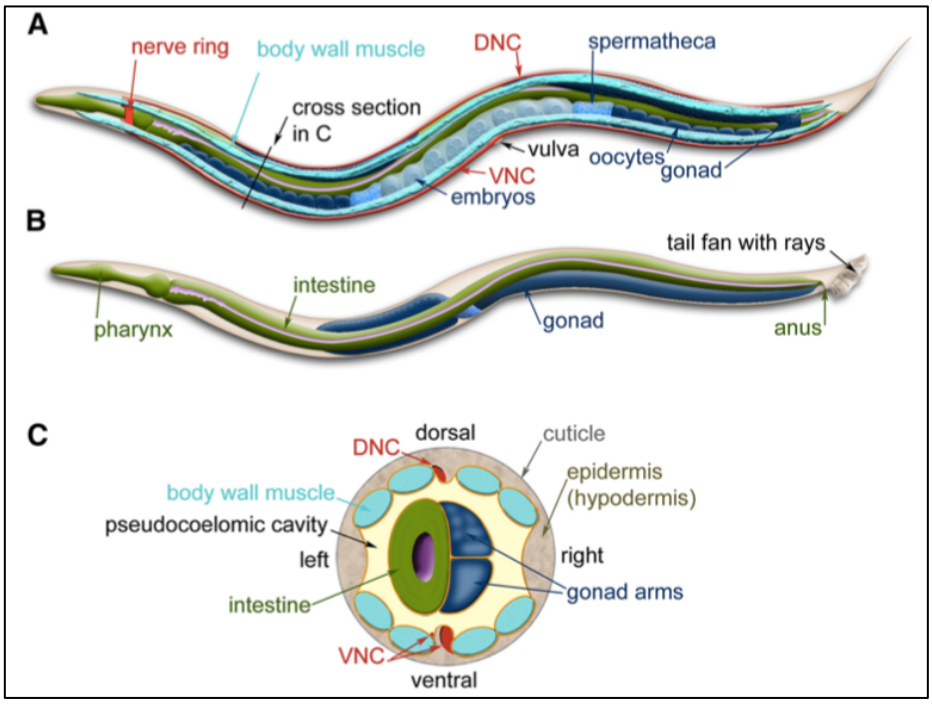

Figure 1. Diagram anatomical systems and structures of both males (**B**) and hermaphrodites (**A**). Cross section of the anterior area of the hermaphrodite, shown by the black arrow in A (**C**) (Corsi et al., 2015).

Life cycle

C. elegans have a short life cycle. Only 3 days incubated at 20°C are needed to become a complete reproductive adult (Corsi et al., 2015). This is a great advantage on the use of *C. elegans* as an animal study, allowing large scale production of synchronized worms within a short period of time. Their life starts during the embryogenesis phase, taking approximately 16 hours at 20°C to be completed (Corsi et al., 2015). In the inside of the hermaphrodites' body, the embryos are produced by self-fertilization, encased in an egg shell and their development is initiated in the uterus (Herman, 2005). At the 30^{th}-cell stage, the eggs are laid through the hermaphrodite's vulva on to their external environment (Herman, 2005). Then the hermaphrodite's embryo hatches with 558 nuclei, becoming in this way, the first larval stage (L1) (Corsi et al., 2015). The larvae undergo four molts totally, with replacements of the cuticles in each step (Riddle et al., 1981). L1, L2 and L3 stages, normally takes about 16h long, the rest near 12h (all at 20°C) (Corsi et al., 2015). When the worm reaches the adult reproductive stage, progeny production can finally start. This begins about 12h from the formation of the L4 molt and continues

for a period of 2-3 days. Once they have utilized all of their self-produced sperm, or if they are fertilized with the male produced sperm, the formation of new progeny have been completed (Corsi et al., 2015). *C. elegans* can then enter a senescence phage. This occurs after their reproductive period and worms can live for several weeks before death (Corsi et al., 2015).

The nematodes can be visible by the naked eye (Hunt, 2017), so using a dissecting microscope up to 100X magnification will allow a clear observation of the whole organism (Corsi et al., 2015). **Figure 2**, shows different *C. elegans* in all of their larval stages. From the embryos form (inside the egg shell) and the possible dauer stage, to the adult reproductive male or hermaphrodite. A clear difference in size through the four larval stages helps identify and classify them into the possible stages, but individual sexes cannot be easily distinguished until the L4 phase (Corsi et al., 2015).

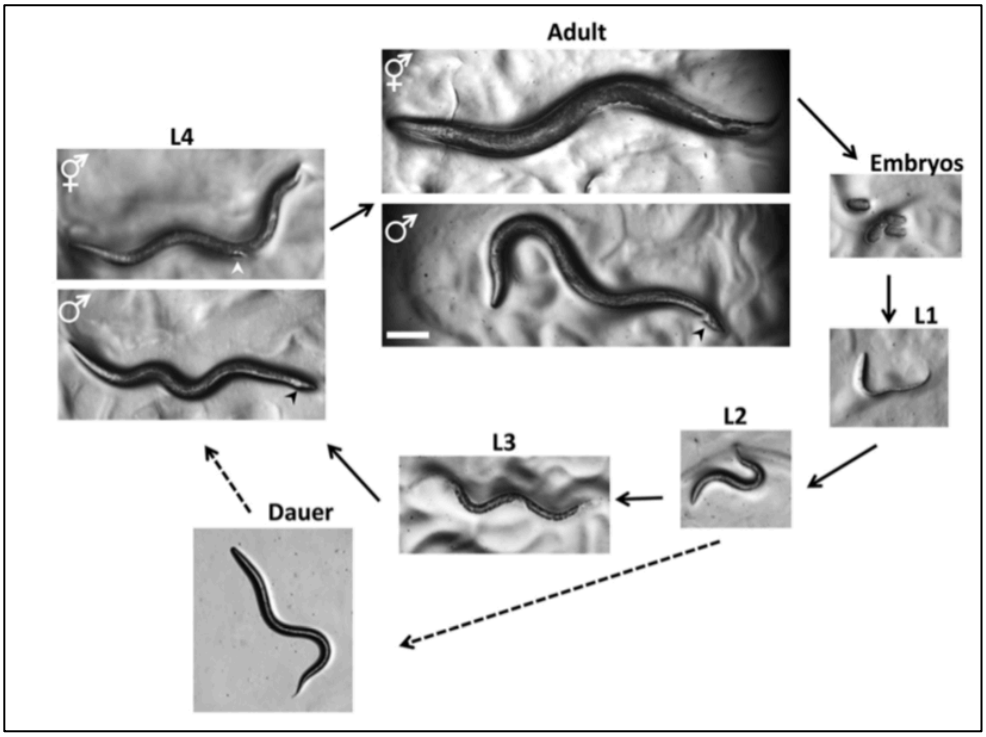

Figure 2. Life cycle of the nematode *C. elegans* in all of its larval stages. At L4, begins to be able to identify males from hermaphrodites. The white arrow shows the developing vulva for the hermaphrodite worm, showing a half circle on the middle of the center of the ventral side. The black arrow shows a wider tail for the male worm. When they become adults, there are clearer distinctions between the two sexes. Hermaphrodites have a thicker and wider body and a tapered tail. Males have a more distinguishable fan-like tail (black arrow). Dauer larvae are thinner than all the normal stages. Photographs taken in the lab on petri dishes, with inoculated bacteria (except for dauer). Bar 0.1 mm (Corsi et al., 2015).

Dauer form

The nematode *C. elegans*, also presents another characteristic making them able to take an alternative route in their life cycle and achieve a “resistant” state. This second molt only appears in response to environmental stress, resulting on the formation of the dauer larvae. During this stage, the nematode presents some morphological and behavioral differences with the normal juvenile, including slimmer body and a fasting diet. Over population, amount of food available at the time, environmental temperature and pH, are some parameters causing the formation of the dauer form (Golden and Riddle,1984). This form is triggered by a pheromone, which prevents the dauer recovery (Golden and Riddle,1984). In spite of, all the studies performed with *C. elegans*, little is known about how this pheromone works, and if it is synthesized in vivo and then released into the environment, or if it binds to and activates a G-protein (Zwaal, 1997; Golden and Riddle,1984). For recovery from this phase into the L4, a heat-stable, hydrophilic “food signal” provides information about the availability of the bacterial food, returning to the normal state (Golden and Riddle,1984).

Dauer larvae also presents a number of morphological and metabolic attributes. During their “resistant” state their oral orifices become almost closed. This closure is caused by an internal plug (**Figure 3**), creating an impediment on their ability to feed on bacteria, therefore, stopping their development. During this state, they can survive for months without any feeding supply (Riddle et al., 1981). They also contain an alae specialized cuticle formed during the second molt (Cassada and Russell, 1975), which provides protection from external influences and changes of the environment (Corsi et al., 2015).

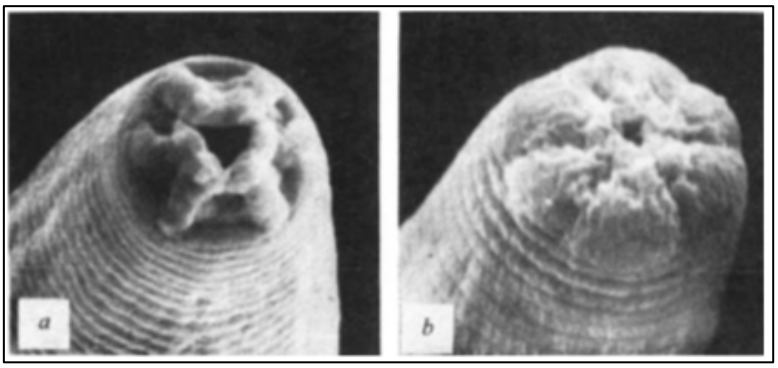

Figure 3. Electron micrograph (with field width of each micrograph of 7.2 μm) of the nose of an L2 *C. elegans* larva (**a**) and the dauer form (**b**). Morphological differences can be identified between both worms, the internal plug completely closes the mouth of the dauer and it has a thinner body (Riddle

In terms of metabolism, at the starting point of their development, during their embryo and L1 form, the glycoxylate cycle is the main route to produce carbohydrates from their stored lipids. Then, during the L2 and the later stages, a metabolic shift undergoes towards aerobic respiration, increasing the activity of the citric acid cycle. Dauers, on the other hand, continue executing the glycoxylate cycle and do not change metabolically (Wadsworth and Riddle, 1989). This means that apparently, the metabolism is regulated during the larval development and that there is a mayor change during the L2, which dauers does not proceed with.

Genetic resemblance with humans

In addition, there are a great number of similarities established between *C. elegans* and higher vertebrate organisms. For example, the genomic sequencing of the whole nematode *C. elegans* (Sequencing Consortium, 1998) brought some light and an unexpected level of gene homology and conservation with vertebrates (Cole et al., 2004), including stress responses in toxicological assays which are observed in higher animal organisms (Leung et al., 2008). A large number of compounds; neurotransmitters, ion channels, synaptic release mechanisms, cholinergic neurons and receptors, including the synthesis, packaging into the synaptic vesicles and their destruction or uptake, have close similarities between nematodes and vertebrates (Bargmann, 1998). So approximately, and depending on the bioinformatics technique used, there are 60-80% of homologue genes between *C. elegans* and humans (Kaletta and Hengartner, 2006). This understanding of their complete genome, also made it possible to create mutant knockout (KO) libraries (Caenorhabditis Genetics Center (CGC) - College of Biological Sciences, n.d.) which provides a great variety of gene suppressed *C. elegans*, to be able to study and perform different kinds of toxicity tests.

Role in toxicity screenings

Many studies have already been performed to evaluate the use of *C. elegans* as a risk assessment model. Reproduction, gene expression and mortality, are some gathered examples shown in **Figure 4**. This represents the most important endpoints that can be measured from the worm, helping scientists to create a bridge between *in vitro* testing, *in vivo* mammalian assays and human exposures (Hunt, 2017).

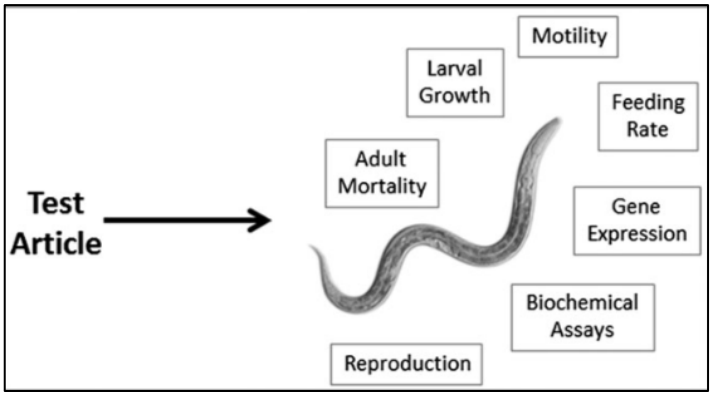

Figure 4. Illustration main *C. elegans* endpoints (Hunt, 2017).

Studies which compare the LD50 obtained from oral rodent and *C. elegans*, have good correlations. This is showed in the early study made by Williams and Dusenbery, testing eight metal salts. The investigation proved an adult mortality correlation of the nematode *C. elegans*, ranking of one-tenth of the cost of the mouse oral test (Williams and Dusenbery, 1988). They also demonstrated, comparing the LC50 of *C. elegans* and the LD50 corresponding to the mammalian test, the nematode results were higher, they concluded that this could be due to the resistant cuticle and characteristic which makes them to be able to live in hasher environments than mouse (Williams and Dusenbery, 1988).

On the other hand, other studies preferred to test directly behavioural characteristics of *C. elegans* and draw conclusions from the EC_{50} regression and the probit values. Anderson et al., found a clear concentration dependency in the inhibition of movement for 7 out of the 9 compounds tested, being chloropyrifos and levamisole the most toxic compounds (Anderson et al., 2004).

There is also an increased number of studies and development of *C. elegans* apparatus analyzers, making it easier for researchers to evaluate a larger number of nematodes and compounds in less time. The Complex Object Parametric Analyzer and Sorter (COPAS; Union Biometrica, Holliston, MA, USA), a revolutionary new apparatus, which uses microfluids and laser-based technologies to analyze hundreds of the *C. elegans* nematodes per minute(Hunt, 2017). This is able to measure LC50 rankings, larval growth and reproductive output. Another measuring apparatus is the WMicrotraker One (Phylumtech), which measures locomotion and development in an easier manner, than the classical touch and movement visual assay.

This studies show that even more investigators from the field of toxicology are starting to show interest in this nematode for extensive assays and predictions. This successful studies gives a first insight on the real potential of the *C. elegans*, not only genetical investigations, but also as a toxicity model (Hunt, 2017)

Acetylcholinesterase enzyme

Acetylcholinesterase (AChE), is an enzyme belonging to the family of cholinesterases. Formed of two different protein domains and about 500 residues, AChE is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, specially at the synaptic cleft or in association with plasma membranes of both pre- and postsynaptic cells (Combes et al., 2001). In addition, it can be found on the red blood cell membranes in humans constituting the Yt blood group antigen (Lionetto et al., 2013). What we can observe at this cholinergic synapses in *C. elegans*, is the neurotransmitter acetylcholine being synthesised by the enzyme choline acetyl transferase, released from the presynaptic side, ultimately hydrolized by the acetylcholinesterase enzyme (Combes et al., 2001), thus AChE prevents a continuous synaptic transmission. Therefore, if AChE is inhibited, the amount of ACh increases, leading to continuous stimulation of the postsynaptic membrane and failure of transmission causing instant death (Combes et al., 2001). It has been demonstrated that 3 genes encode this enzyme in nematodes: *ace-1, ace-2, ace-3* (Johnson et al.,1988). Another gene has been already identified, *ace-4*, but the protein it encodes, does not seem to be active (Grauso et al., 1998). Each of these genes encodes different AChE that can be located on different tissues inside the body of a *C. elegans* (Combes et al., 2001). For example, *ace-1* is expressed in muscle cells and a few neurons, whereas *ace-2* is mainly expressed in motoneurons. *ace-3* represents a minor proportion of the total AChE activity and is expressed only in a few cells (Combes et al., 2001). ACh-mediated behaviours have been established, being locomotion the most relevant, involving neuromuscular and nerve-nerve transmissions and by far the most number of cholinergic neurons (Rand, 2007). Other behaviours mediated by AChE activity are egg laying, pharyngeal pumping, defecation cycling and male mating (Rand, 2007). In this way, a hypothesis can be dragged; if AChE is inhibited by a toxicological agent in *C. elegans*, then it would be reflected on the locomotion of the nematode, causing paralysis and even death.

Organophosphates

Organophosphate (OP) pesticides are widely used for pest control on crops in agriculture and livestock, including domestic and garden applications (Lionetto et al., 2013). Chlorpyrifos (O ,O -diethyl O -3,5,6-trichloro-2-pyridyl thiophosphate) and profenofos [O-(4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate] are examples of organophosphate pesticides used frequently around the world. One of the main differences between both types of pesticides is that the parent form of profenofos is active and chloropyrifos needs to undergo bioactivation in vivo to a more potent inhibitor (Sams et al., 2004). The cytochromes P450 (CYP) is shown to be the responsibles for this bioactivation, mediating the formation of a phosphooxythiran intermediate, resulting in either a desulphuration reaction, producing the oxon or a derylation reaction that detoxifies the organophosphate to hydrolysis products (**Figure 5**) (Sams et al., 2004).

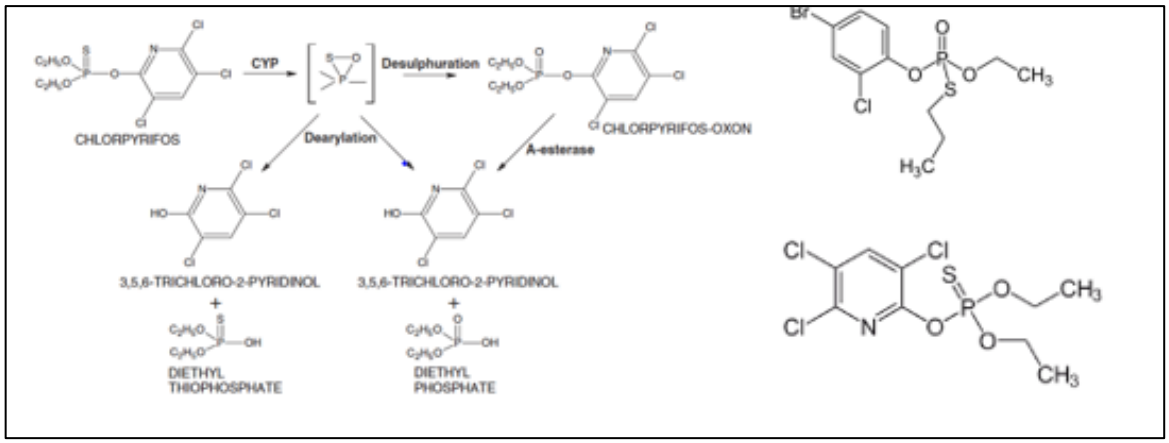

Figure 5. Bioactivation pathways of chlorpyrifos (CPF) (left), chemical structure of CPF (bottom right) and profenofos (PFF) (top right) (Sams et al., 2004).

OBJECTIVES

OBJECTIVES

The purpose of this investigation is to evaluate the toxicity of chlorpyrifos-oxon and profenofos on the *C. elegans* model.

As secondary objectives:

- Evaluate the potential use of *C. elegans* as an animal model in toxicity testing.
- Testing of the WMicrotracker ONE instrument for measurement of the locomotion of the nematodes in liquid medium.
- Create a platform for culturing and maintenance of *C. elegans* N2 strain ready to be used in further research.

MATERIALS AND METHODS

MATERIALS AND METHODS

Nematode culture and exposures

Caenorhabditis elegans, were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). Wild type strain N2, was cultured in Nematode Growing Medium (NGM) agar plates, incubated at 20ºC and seeded with *E. coli* OP50 strain (a uracil-deficient strain of *Escherichia coli*), as a food source. Plates were changed every 3 days to prevent overpopulation and sufficient food was provided to prevent the dauer formation (Stiernagle, 1999).

Before the exposure, age-synchronous adults from eggs of emergent dauers larvae were produced. (Donkin and Williams, 1995). This protocol involved bleaching (1mL 1M NaOH, 2.75mL dH_2O and 1mL 4% NaOCl) 3 day gravid nematodes and incubating them in M9 without any food, overnight. Then when they reached the L3 stage, the *C. elegans* were rinsed with K-medium, at least three times to get ready for testing. The measurements were made using a 96 wells plate in K-medium (0,032M KCl and 0,051M NaCl) with a rich amount of *E. coli*, to make sure that starvation and consequent dauer formation does not affects the results (Cole et al., 2004).

As the organophosphates (OP) were diluted in ethanol (final concentration 0,2%), a group exposure to ethanol was made. A control group and 8 different concentrations of chlorpyrifos and profenofos were finally assessed, noted in **Table 2-3**. Solutions of nematodes were adjusted to obtain 5 worms/10µL. Approximately 50 worms were used in each well and then 3 readings were made; a basal rate, a 2-h exposure and a 24-h exposure. A 48-h readings were assessed, but no movement was recorded.

Locomotion, the endpoint examined was measured by the WMicrotracker ONE (Phylumtech). This machine detects animal movement through infrared microbeam, light scattering. Each well in liquid medium is crossed by at least one infrared microbeam, scanned more than 10 times per second. This allowed to measure the average movement in each well creating a cause-consequence relationship between the different concentrations of OPs and the amount of ACh enzyme inhibited.

First, the dilutions for the profenofos and chlorpyrifos-oxon were prepared, with final concentrations noted in **Table 2-3**. Then, in the 96 wells plate, 2 control groups and 2 test groups were performed. Ethanol was also used as the organic solvent (final concentration 0.2%). The enzyme was adjusted to finally have 1 U/mL in each well. After the 15 minutes incubation of the pesticide and the enzyme, acetylcholine (ATC) and Ellman's reagent (DTNB) was added to each well. ATC will be hydrolyzed into acetate and thiocholine by the non-inhibited fraction of the enzyme and the indicator DNTB would form a yellow complex with the thiocholine produced, as shown in **Figure 6**. This yellow substance which will be measured with the spectrophotometer at 412nm wavelengh (37°C).

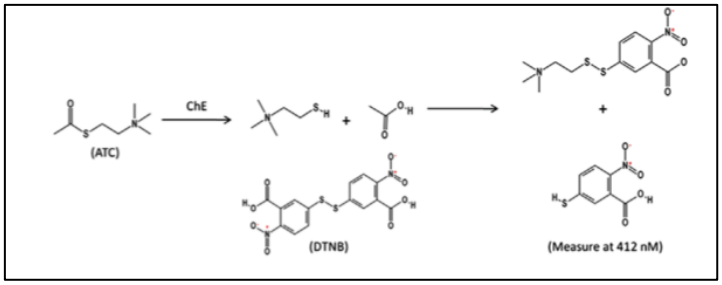

Figure 6. Working method of DTNB, thiocholine production (McGarry et al., 2013)

Table 2a. Tested concentrations (mM) for both assays and

		CONCENTRATION (mM)				
		1	**2**	**3**	**4**	**5**
AChE assay	**Chlorpyrifos-oxon**	0	5×10^{-6}	1×10^{-5}	2.5×10^{-5}	5×10^{-5}
	Profenofos	0	5×10^{-8}	1×10^{-7}	5×10^{-7}	1×10^{-6}
Nematode assay	**Chlorpyrifos-oxon**	0	1×10^{-4}	5×10^{-4}	1×10^{-3}	5×10^{-3}
	Profenofos	0	1×10^{-4}	5×10^{-4}	1×10^{-3}	5×10^{-3}

Table 2b. Tested concentrations (mM) for both assays and OPs.

		CONCENTRATION (mM)						
		6	**7**	**8**	**9**	**10**	**11**	**12**
AChE assay	**Chlorpyrifos-oxon**	1×10^{-4}	2.5×10^{-4}	5×10^{-4}	1×10^{-3}	2.5×10^{-3}	5×10^{-3}	2×10^{-2}
	Profenofos	2.5×10^{-6}	5×10^{-6}	1×10^{-5}	2.5×10^{-5}	5×10^{-5}	1×10^{-2}	
Nematode assay	**Chlorpyrifos-oxon**	1×10^{-2}	5×10^{-2}	1×10^{-1}	5×10^{-1}			
	Profenofos	1×10^{-2}	5×10^{-2}	1×10^{-1}	5×10^{-1}			

Statistical analysis

The statistical analysis included 3 repeat plates for both the AChE assay and the *C. elegans* assay. For the nematode assay, movement for each treatment was calculated as a percentage of the control group movement. A non-linear curve dose-response for the 24-h exposure was created for each pesticide using Graph Pad® and obtaining EC_{50}values. Through Probit analysis (Excel) EC_{50} values were also generated.

The AChE assay, was analysed by Graph Pad® and non-linear curve dose-responses were generated, obtaining the IC_{50} values of each OP.

RESULTS

RESULTS

C. elegans assay

Prior to analyze the data, a t-test (two-sample, unequal variance) confirmed that statistically, the control group and the ethanol group had similar results, showing that the organic solvent with 0.2% concentration, had any effect on the nematodes (α= 0.05, t-test= 0.257). From this information, comparisons with the control group were performed to see how these OPs had affected on the movement of the *C. elegans*. A series of bar graphs were performed for the basal rate, 2h and 24h exposure, for both OPs. The graphs are analyzing the percentage of movement for each concentration and being adjusted for the 100% movement control. As shown in **Figure 8, 9** and **10**, for profenofos, after 2 hours of exposure, there is a slight decrease on movement of the *C. elegans* after 2 hours of exposure. For the 24 hours, there is a great decrease, but this is only noticeable from the 0.001mM of chloropyrifos-oxon onwards, the values before have increased. On the other hand, in **Figure 11, 12** and **13,** for chloropyrifos-oxon, after 2 hours of exposure, there is a decrease of percentage of movement for the more concentrated values, and an increase for the rest of the values. For the 24 hours exposure, a decrease in movement appeared, more noticeable for the concentrated values. The EC_{50} values obtained through Probit analysis were, 0.0039mM for PPF (P-value= 1.91×10^{-6}) and 0.00068mM for chlorpyrifos-oxon (P-value= 8.07×10^{-7}).

A dose-response curve was also performed as shown in **Figure 14** and **15** , obtaining EC_{50}= 0,001mM for profenofos and EC_{50}= 0,0025 for chloropyrifos-oxon.

Another important feature obtained, is the standard deviation and the protocols developed. For concentrations of both of the pesticides, values were notably high. This was generated, as the raw readings obtained varied greatly between plates. The protocols gave rise to standard *C. elegans* culturing procedures, as seen in **Figure 7**.

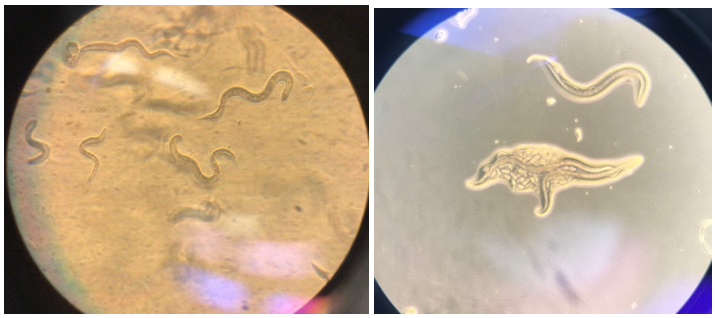

Figure 7. Pictures performed in the lab. Various larval stages

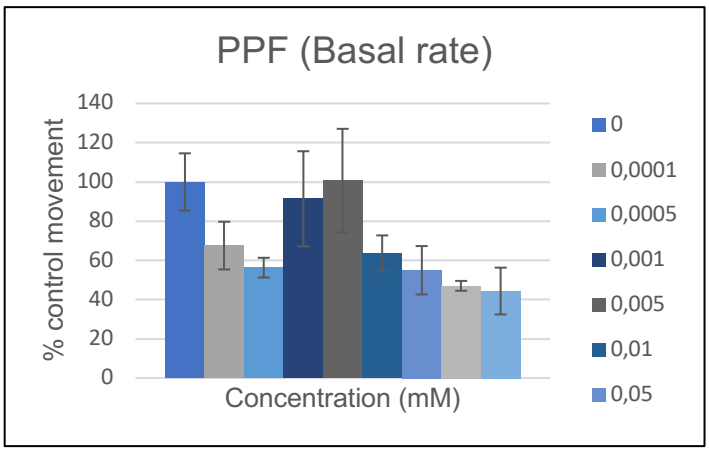

Figure 8. Bar chart for profenofos, percentage movement for each concentration at basal rate.

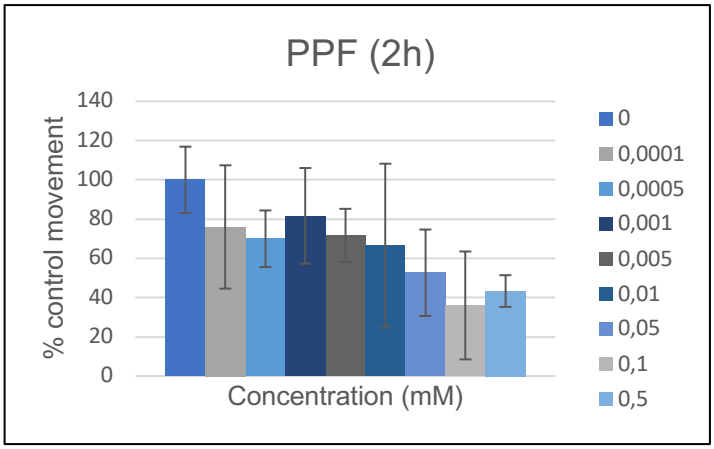

Figure 9. Bar chart for profenofos, percentage movement for each concentration at 2 hours of exposure.

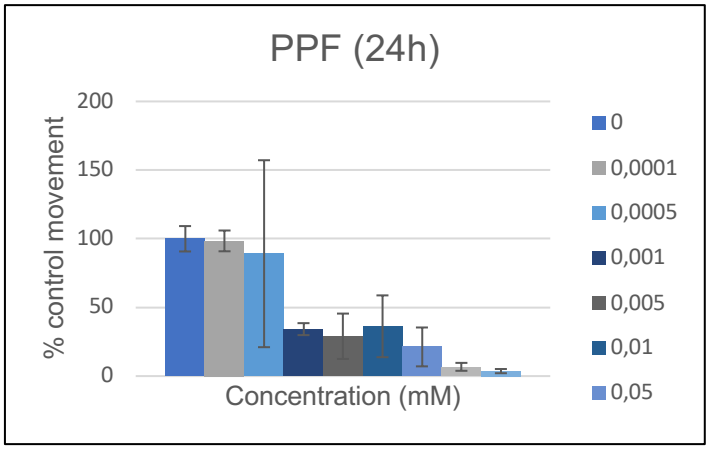

Figure 10. Bar chart for profenofos, percentage movement for each concentration at 24 hours of exposure.

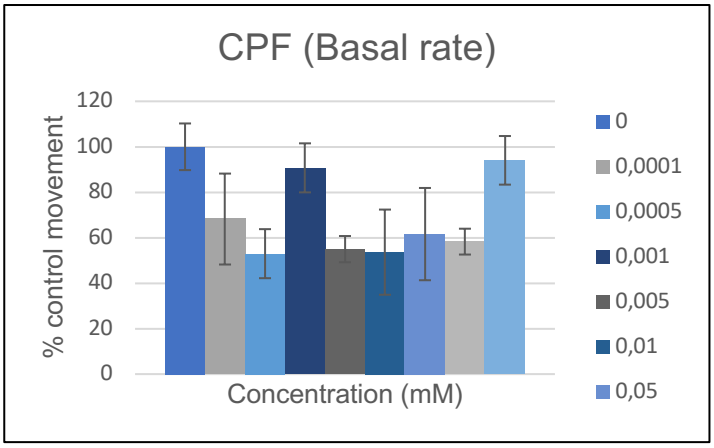

Figure 11. Bar chart for chloropyrifos-oxon, percentage movement for each concentration at basal rate.

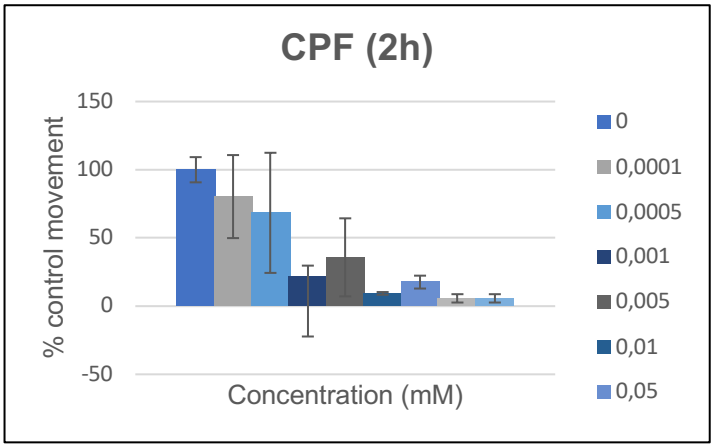

Figure 12. Bar chart for chloropyrifos-oxon, percentage movement for each concentration at 2 hours of exposure.

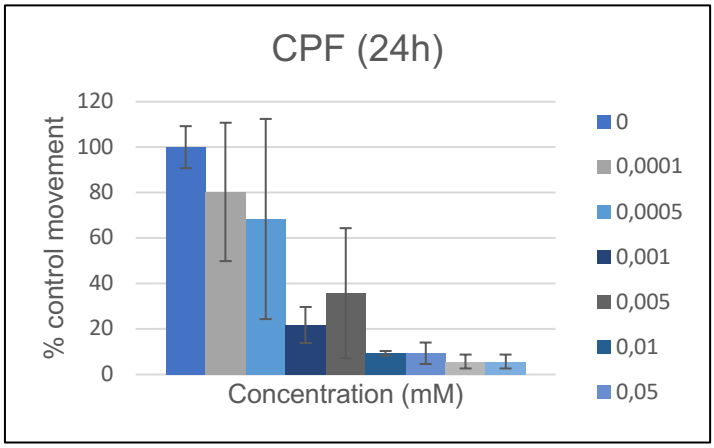

Figure 13. Bar chart for chloropyrifos-oxon, percentage movement for each concentration at 24 hours of exposure.

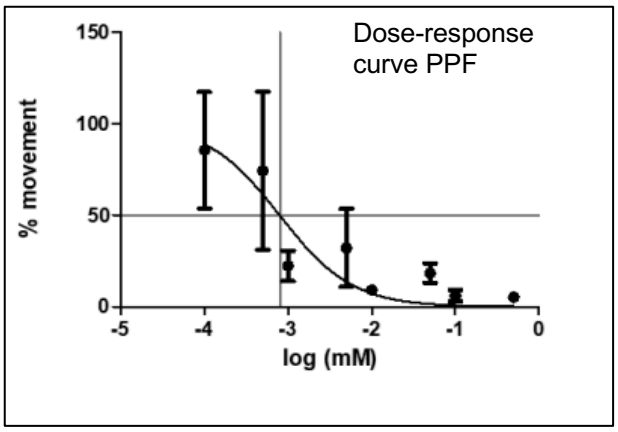

Figure 14. Dose-response curve for profenofos (*C. elegans*).

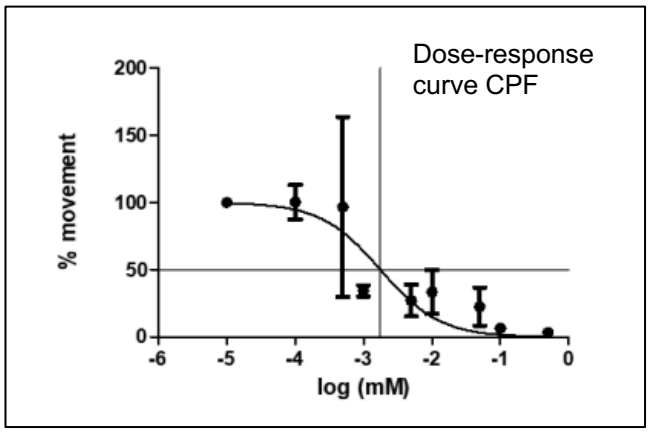

Figure 15. Dose-response curve for chloropyrifos-oxon (*C. elegans*).

AChE assay

The curve dose response for the second assay also showed a clear concentration dependent inhibition of the enzyme inhibition. The IC_{50} were also calculated, 588.1mM for profenofos and 5.278mM for chlorpyrifos-oxon. The dose-response curve generated is shown in **Figure 16** and **17.** A comparison between the dose-response generated values, such as EC_{50} and IC_{50} is shown in **Table 3.**

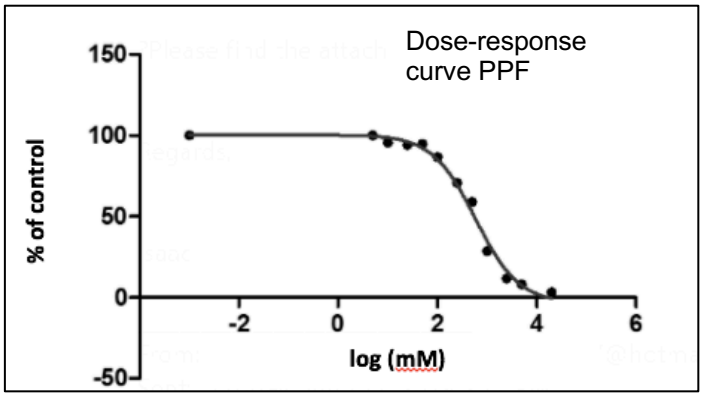

Figure 16. Dose-response curve for profenofos (*in vitro*).

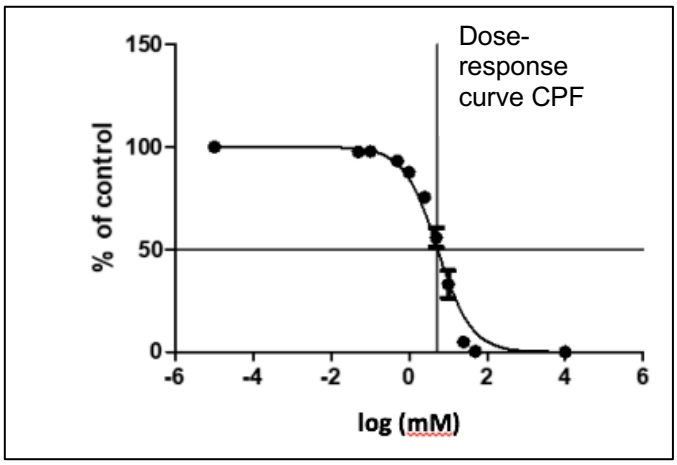

Figure 17. Dose-response curve for cloropyrifos-oxon (*in vitro*).

Table 3. Comparison results EC_{50} and IC_{50} for both pesticides and both conditions (curve dose-response).

EC_{50}/IC_{50} (mM)- Curve dose-response		
	Cholopyrifos-oxon	Profenofos
***In vivo* (*C. elegans*)**	0.0025	0.001
In vitro	5.278	588.1

Discussion

Discussion

The goal of the work reported here, is to evaluate the use of *C. elegans* for toxicity assays by the determination of sensitivity to two different organophosphates. Results obtained through the bar charts, recorded a gradual decrease in movement, having an acute toxicity after 24 hours of exposure, and a slight significance after 2 hours of exposure. If a comparison is performed with the investigation carried out by Roh & Choi. They perceived a decline in motion of the nematode by the chlorpyrifos action, after 24 hours of exposure (Roh & Choi, 2008). Other studies, like Anderson at al., also studied the relationship between the organophosphate chlorpyrifos a the reduction of locomotion of the worm. Results viewed a slight decrease in behaviour after 4 hours of exposure, having more acute effect after 24 hours (Anderson et al., 2004). This suggests, than the investigation performed, with only 2 hours of exposure, was not sufficient for a complete inhibition of the AChE inside the nematode's body. This may be due to the fact that when working with a whole organism, the rate of diffusion into their body is lower, and compared with the *in vitro* technique, more incubation time is needed.

On the other hand, the results obtained for the standard deviation analysis suggests that the investigation perform shows a large fluctuation a low reliability. The solutions performed for preparing 50 worms per well, have low accuracy, developing into the high standard deviation. A better sampling preparing method is needed to be performed, including more than 3 repeats per condition, to lower that difference in results.

The high standard deviation can then explain the difference of EC_{50} values obtained by this study and the EC_{50} values obtained by other investigations. An EC_{50}= 0.005mM for chlorpyryfos in Andersons et al., compared with the result obtained in this work, 0.00068mM, through probit analysis. This difference in results may also be due, to the fact that chlorpyrifos-oxon was used in this investigation, and on Andersons et al, it is not specified. This difficult the possible comparisons between studies.

In spite of, the low successful results in the in vitro assay, the production of standardized protocols for incoming investigations was achieved. Maintenance, cleaning protocols and age-synchronous preparation of nematodes were performed in the laboratory and developed using the information provided by the Wormbook web page. The whole protocols are included in the Appendix section.

Finally, as a secondary objective, the *in vitro* technique resulted in a reliable and low standard deviation values. As it is indicated in Sams et al. investigation, the bioactivation of chlorpyrifos results in a 100-fold more potent inhibition, and for this reason we obtain a much lower IC_{50} values for tchlorpyrifos-oxon (5.278mM), compared with profenofos (588.1mM).

Conclusion

Conclusions

1. An initial evaluation test of the effect of *organophosphtes* on *C. elegans was performed.*

2. *The* prefenofos and chloropyrifos-oxon chemicals assayed showed some effect on the nematodes but results obtained have a high standard deviation and are not completely reliable.

3. A laboratory platform for maintenance and testing preparation for *C. elegans* was developed with success.

4. The new laboratory instrumental *WMicrotraker One* to measure the locomotion of nematodes was successfully implemented.

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Appendix

Appendix

Preparation of Nematode Growing Medium (NGM) petri plates and seeding

Created by: Maria Gregori Balaguer
Version: 1.2 (20190315)

The following protocol is based on the preparation of the Nematode Growing Medium (NGM) and then the seeding process with the *E. coli* OP50 we have cultured before. Different plate sizes can be used, but 90mm diameter plates are useful for strain maintenance and they will have extra space. Use Parafilm to seal plates.

Materials:

- 3g NaCl, 17g agar, 2.5g peptone, H2O to 1L (for preparation of 1L of NGM)
- Autoclave
- 2 liter Erlenmeyer flask
- 55°C water bath
- 1ml 1M CaCl2, 1ml 5mg/ml cholesterol in ethanol, 1ml 1M MgSO4, 25ml 1M KPO4 buffer
- 90mm diameter sterile petri dishes
- *E. coli* liquid culture
- Glass rod
- air-tight container (like an air-tight plastic bag to store the plates)
- Bunsen burner

Methods:

1. Mix 3g NaCl, 17g agar, and 2.5g peptone in a 2 liter Erlenmeyer flask. Add 975ml H2O. Cover mouth of flask with aluminium foil. Autoclave for 50 min (to).
2. Cool flask in 55°C water bath for 15 min.
3. Add 1ml 1M CaCl2, 1ml 5mg/ml cholesterol in ethanol, 1ml 1M MgSO4 and

   25ml 1M KPO4 buffer. Swirl to mix well.

4. Dispense the NGM solution into the sterile petri plates (set up a Bunsen burner).

   Fill plates 2/3 full of agar.

5. Leave plates at room temperature for 2-3 days before use to allow for detection

   of contaminants, and to allow excess moisture to evaporate. Plates stored in an air-tight container (like an air-tight plastic bag) at room temperature will be usable for several weeks.

6. For the seeding, using sterile technique, apply approximately 0,1 ml of *E.coli* OP50 liquid culture to the medium NGM plates (3 drops).
7. If desired, the drop can be spread using the pipet tip or glass rod. Spreading will create a larger lawn, which can aid in visualizing the worms. Take care not to spread the lawn all the way to the edges of the plate; keep the lawn on the center. The worms tend to spend most of the time in the bacteria. If the lawn

extends to the edges of the plate, worms may crawl up the sides of the plate, dry out and die.

8. Allow the *E. coli* OP50 lawn to grow overnight at 37°C for 8 hours (cool plates to room temperature before adding worms).
9. Seeded plates stored in an air-tight container will remain usable for 2-3 weeks.
10.

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Cleaning contaminated *C. elegans* stocks

Created by: Maria Gregori Balaguer
Version: 1.2 (20190318)

The following protocol is based on different methods of cleaning contaminated *C. elegans* stocks. The moist and rich in minerals environment of the worm cultures gives an ideal opportunity for bacteria, yeast or mould to grow. Most contaminants will not hurt the worms, but they need to be eradicated as results may be affected. Mould can be removed by chunking and serially transferring, allowing the worms to craw away from the contaminant (protocol 1). Bacterial contaminants and yeast are easily removed by treating with a hypochlorite solution, which will kill the contaminant as all worms that may not be protected by the egg shell. This can be done using an entire plate that is contaminated (protocol 2), or it can be done quickly using a single hermaphrodite (protocol 3). Decide which protocol adjusts to your case, depending on the purpose of the cleaning.

Protocol 1(removing mould contaminants from *C. elegans* stock plates):
Materials:

- Scalpel
- Contaminated culture
- NGM petri dish and seeded with E. coli OP50 (new clean dish)
- Worm picker
- Bunsen burner

Method:

1. Sterilize a scalpel or spatula in a flame and remove a chunk of the agar from the contaminated plate. Remove the cover of the contaminated plate only as long as necessary.
2. Place the chunk of agar at the edge of a seeded clean plate. Allow the worms to crawl out of the chunk and across the *E. coli* OP50 lawn to the opposite side of the plate. The worms are attracted by the *E. coli* food source.
3. Once the worms have reached the other side of the plate, pick individual animals with a worm picker and place it on the edge of another clean plate.

Protocol 2 (egg prep: removing bacterial or yeast contaminants from *C. elegans* stock plates):
Materials:

- 0,5 ml 5N NaOH
- 1 ml Household bleach (5% solution of sodium hypochlorite)
- Contaminated culture
- Sterile H2O
- 5ml conical centrifuge tube
- vortex
- 2 NGM plate seeded with an *E. coli* OP50 lawn
- Table top centrifuge
- Pasteur pipette

Method:

1. Use contaminated C. elegans stock plates that have many gravid hermaphrodites (embryos that can be found inside the egg shells will survive this procedure). Wash the plates with sterile H2O. Pipette the H2O across the plate several times to loosen worms and eggs that are stuck in the bacteria.
2. Collect the liquid in a sterile 5ml conical centrifuge tube with cap. Add H2O to total 3,5 ml.
3. Mix 0,5 ml 5N NaOH with 1ml bleach (add NaOH to weaken the bleach, to make sure that it don´t affect the embryos in the egg shells). Make this solution fresh before use! Add to the centrifuge tube with worms.
4. Shake well or vortex the tube a few seconds. Repeat shaking/vortexing every 2 minutes for a total of 10 minutes.
5. Spin the tube in a table top centrifuge for 30 seconds at 1300x g to pellet released eggs.
6. Aspirate to 0,1 ml of the supernatant.
7. Add sterile H2O to 5 ml. Shake well or vortex for a few seconds.
8. Repeat steps 5 and 6.
9. Use a Pasteur pipet to transfer the eggs in the remaining 0,1 ml of liquid to the

   edge of a clean NGM plate seeded with an E. coli OP50 lawn.

10. The next day the eggs will have hatched and the larvae will have crawled into the

    E. coli OP50 lawn. Transfer the hatched larvae to a clean NGM plate seeded with an *E. coli* lawn.

Protocol 3 (egg prep in a drop: small scale method for removing bacterial or yeast contaminants):
Materials:

1:1 NaOH: bleach mixtures
2 clean NGM plate seeded with E. coli

Gravid *C. elegans* hermaphrodites **Method:**

1. Make a 1:1 mixture of 1N NaOH:bleach. Put a drop of this solution on the edge of a clean NGM plate seeded with an *E. coli* OP50 lawn.
2. Put several gravid hermaphrodites in the drop. The solution will kill the contaminants and hermaphrodites but will soak into the plate before the embryos hatch.
3. The next day the larvae will have crawled into the E. coli OP50 lawn. Transfer them to a clean NGM plate seeded with an *E. coli* OP50 lawn.

References

1. Stiernagle, T. (1999). Maintenance of C. elegans. *C. elegans*, *2*, 51-67.

Transferring *C. elegans* from one petri dish to another

Created by: Maria Gregori Balaguer
Version: 1.2 (20190317)

The following protocol is based on the different methods for transferring the *C. elegans* from one petri dish to another. Two methods are available, depending on the amount of worms that wants to be transferred. The first method explains how to transfer many worms at once. The second method explains the best way to transfer one or even two worms at once using a worm picker. A binocular lens microscope may help to visualize the worms during the process.

Protocol 1: Materials:

- Scalpel
- Petri dish with C. elegans (old dish)
- Petri dish with medium and seeded with *E. coli* (new dish)

Method ("chunking"):

1. Using a sterilized scalpel, move a chunk of agar from the old plate to a fresh plate. There will be hundreds of worms in the chunk of agar.
2. The worms will crawl out of the chunk and spread out onto the bacterial lawn of the new plate

Protocol 2: Materials:

- 1-inch piece of 32 gauge platinum wire
- Bacteriological loop holder
- Hammer
- *E. coli* OP50 liquid culture
- Petri dish with C. elegans (old dish)
- Petri dish with medium and seeded with *E. coli* (new dish)
- Microscope (stereoscopic microscope)

Method 2 (transferring individual worms):

1. A worm picker needs to be made. It can be made by mounting a 1-inch piece of 32 gauge platinum wire into a bacteriological loop holder.
2. Platinum wire heats and cools quickly and can be flamed often (between transfers) to avoid contaminating the worms stock (make sure that the wire is cooled before picking up the worms). The end of the wire, used to pick up the worms, can be flattened slightly with a hammer. Avoid poking holes in the agar, as worms crawl into the holes, making it difficult to see or pick them.
3. To pick a worm identified under the microscope, slowly lower the tip of the wire and gently swipe the tip at the side of the worm and lift it up. Another method is to get a blob of *E. coli* OP50 on the end of the picker before gently touching it to the top of the chosen worm. The worm will stick to the bacteria. Several worms can be picked by this method, although worms left too long on the pick will desiccate and die.
4. To put a picked worm on a fresh plate, slowly lower the tip on the worm picker, gently touch the surface of the agar, and hold it there to allow the worm to crawl off the picker.

5. This technique is complicated and it may be difficult as first. It is important to be gentle and calm when capturing the worms, practice will make this method become easier.

References

1. Stiernagle, T. (1999). Maintenance of C. elegans. *C. elegans*, *2*, 51-67.

Use of Caenorhabditis elegans based assays as an alternative strategy in toxicity testing

Obtaining age-synchronous cultures of *C. elegans*

Created by:	Maria Gregori Balaguer
Version:	1.1 (20190422)

The following protocol is based on how to produce synchronous *C. elegans* cultures. The aim is to produce a culture were all of the *C. elegans* are at the same larval stage. This may be useful for the testing protocols, as different larval stages produce different readings.

Materials:

- M9 Buffer (3g KH2PO4, 5g NACl, 1ml 1M MgSO4, H2O to 1 litre)
- Axenized C. elegans eggs from steps 4-8 (cleaning contaminated C. elegans stocks, protocol 2 (egg prep: removing bacterial or yeast contaminants from *C. elegans* stock plates))
- S Basal (5.85 NaCl, 1g K2HPO4, 6g KH2PO4, 1ml cholesterol (5 mg/ml in ethanol), H2O to 1 litre)
- *E. coli* OP50 liquid culture
- 1-2 litre flask
- Incubator 20°C
- 50 ml sterile conical centrifuge tube
- Centrifuge

Method:

1. Use contaminated *C. elegans* stock plates that have many gravid hermaphrodites (embryos that can be found inside the egg shells will survive this procedure). Wash the plates with M9 buffer. Pipette the M9 buffer across the plate several times to loosen worms and eggs that are stuck in the bacteria. Introduce the worms in a 5mL centrifuge tube.
2. Centrifuge for 2 minutes at 400xg (~1500 rpm on a standard table centrifuge) at room-temperature and discard supernatant.
3. Perform 1-3 washes until the buffer appears clear of bacteria.
4. Prepare a solution of 1mL of sodium hypochlorite ~4%, 1.25mL 1M of sodium hydroxide, 2.75mL of dH2O. Add the solution to the centrifuge tubes (about 1-2 mL) and agitate for 3-9 minutes.
5. Stop the reaction by adding M9 buffer to fill the tube.
6. Quickly centrifuge (since treatment may still be active) for 1 minute at 400 x g and discard supernatant.
7. Wash pellet three more times by filling the tube with M9 buffer.
8. Centrifuge for 2 minutes at 400xg.
9. Aseptically transfer the axenized eggs (from step 8 (egg prep: removing bacterial or yeast contaminants from *C. elegans* stock plates)) to a 250 ml M9 Buffer in a 1-2 litre flask and allow to incubate overnight at 20°C suing fairly vigorous shaking to obtained L1 animals.
10. Overnight introduce the worms in a shaking incubator at 20˚C without any food.
11. Mid-L1 larvae can be harvested after approximately 8 hours, mid-L2 larvae at approximately 18 hours, mid-L3 larvae at approximately 25 hours adn mid-L4 larvae at approximately 37 hours.

References:

1. Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., & Cerón, J. (2012). Basic Caenorhabditis elegans methods: synchronization and observation. *JoVE (Journal of Visualized Experiments)*, (64), e4019.
2. Stiernagle, T. (1999). Maintenance of C. elegans. *C. elegans*, *2*, 51-67.