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Bioaccumulation and estrogenic effects of DDT, Arochlor 1254 and their 1:1 mixture on zebrafish (*Brachydanio rerio*).

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Bioaccumulation and estrogenic effects of DDT, Arochlor 1254 and their 1:1 mixture on Zebrafish (*Brachydanio rerio*)

Abtract

The rate of accumulation, kinetic of elimination and the metabolism way of many chlorinated hydrocarbons (e.g. DDT, PCBs) have been in the last year sufficiently cleared up. Until today, their effects against the specific organisms were still a subject of controversial discussions. It was also the case for potential endocrine effects to influence the spermatogenesis correlated with possible changes of the population's vitality. Through a critical Literatur-Recherche, evidence is produced with special regard to substance mixture (e.g. PCBs) that as a matter of fact, the analysis of residues was not strongly discussed. However because of the lack of sufficient standard quality of laboratory safety mechanisms, the ecoeffects were scarcely taken into consideration

Substances with estrogenic effects are from special interest naturally ecosystematic. To clear this situation, three questions could be at the centre of attention:

1. Do the chemicals cause a special harmful effect of the male reproductive tract?
2. Could some particular chemical mixtures act to bind and activate the human estrogen receptor (hER)?
3. Are the life stages of an organism specially sensitive to the effects of chemicals and therefore be established as Screening- Test- System?

In the present study, the connected effects of DDT and Arochlor 1254 (A54) as single substance and in 1:1 mixture according to their estrogenic effectiveness on zebrafish (*Brachydanio rerio*) were therefore investigated.

For that, the short and long time exposure tests with the different tested concentrations and mixture of the chlorinated hydrocarbons were first of all checked.

The concentrations of the tested substances and their mixture ranged between 0.05 µg/l and 500 µg/l and separated by a factor of 10. It was turned out that the test concentrations of 500 µg/l were too toxic to zebrafish in all the cases. The LC50 values of DDT, A54 and the chemical mixtures were determined. The analysis of all the test concentrations showed that the rate of mortality was higher during the exposure to the chemical mixtures. Therefore the amount of chemicals absorbed by zebrafish was checked. For that the static fish test "305D" of the OECD guidelines for testing of chemicals was used (Bioaccumulation test). Afterwards according to the data established, the experiment was followed up with four concentrations of DDT, A54 as well as their 1:1 mixture anew each separated by a factor of 10 and ranging

between 0.05 µg/l and 50 µg/l. The Bioaccumulation test within 8 days showed that the zebrafish really picked up and accumulated the chemicals, but in all the cases no equilibrum was reached. The concentration of 0.05 µg/l was beared by the zebrafish before the end of the eighth day, later the chemicals were not more detectable (NOEC-values). At the other tested concentrations, the bioaccumulation factor within eigth days (BCF8days) increased with higher levels of the tested chemicals. The values of the BCF8days tests demonstrated that the zebrafish absorbed more DDT than A54.

Putting up on these analyses, the experiment was followed by the investigation of the life cycle (LC) of zebrafish. First of all, the eggs were exposed to the different tested concentrations of both chemicals and their mixture. It was turned out that the chemicals caused significant changes in the rate of hatchability and the reproduction and also influenced the length of juvenile fish. These three parameters were tightly correlated with the chemical content. In all the cases, no adult fish survived at the concentration 50 µg/l. Over and about that there was a deviation between the expected and the experimental durations of the life cycle stages (LCS), which were found delayed for few to many days according to the levels of the pesticides. The investigation of the life stages up to 6 weeks in the F-II generation demonstrated that the development of the life cycle stages lasted longer when the mixture of the chemicals was tested, than when the chemicals were tested alone and also that the fish seemed to tolerate more A54 than DDT.

During this investigation, the length of fish emerged were evaluated on fixed spcimens. By means of the Analysis of Variance (ANOVA) to compare the results, the F-ratio at degree of freedom (4,40) were 1.37, 1.65 and 0.88 for DDT, A54 and their mixture respectively. The length of fish was found more reduced with higher concentrations and more significant when the fish emerged in test chambers which were contaminated with the chemical mixture. The reduction in the rates of hatchability, adult survival and the population size, the retardation of the developmental stages of the LC and the inhibition of growth of test organisms by the chemicals and their mixture could have been due to the disorders that they caused in the male reproductive tract. That is why the quality, quantity and the duration of the activity of sperm released by the male zebrafish exposed to the different tested chemicals during mating events within a certain period were investigated using the methods of Leong (1988) and Shapiro et al (1994). The samples were collected gradually after 24 hours, 2 weeks, one and two months of exposure to the different tested concentrations of the chemicals. They showed that the decrease in sperm counts registered was closer to be significant with long time exposure of fish to the pesticides. The levels of the chemicals and the exposure time reduced thereafter the

number, activity and life span of sperm released by the treated male fish. These reductions were more significant when the test organisms were exposed to the pesticide mixture.

To know more about the causes of sperm degeneration, the examination of the spermatogenesis and the structure of the testes were carried up. For this, the testes were embedded in EPON 812 resin and cut in semi and ultrathin sections which were observed under light and electron microscope respectively.

Under light microscope, a reduction of the spermatids content in the lobule lumen of the testes was clearly demonstrated. This reduction in the number of the spermatids increased with higher tested concentrations and longer exposures of fish to the chemicals. As well, the efferent ducts were found compressed by the effects of chemicals leading to the enlargement of the interlobular space which become more delimited and bigger as the tested concentrations increased. At this level , the investigation was followed by seeking the causes of the reduction of the late stage of spermatogenesis with the electron microscope. It was observed that after 2 months of exposure to the chemicals, in the late stage of spermatogenesis were mostly the primary spermatids present, very few and sometimes no secondary (SpII) and tertiary spermatids (SpIII) which consequently reduced also the number of mitochondria. Thereafter, it was found out that there was no heterophagic vacuoles whose presence in the cytoplasm is a sign of phagocytic activity for germinal components.

In general, the absence of phagocytizing activities which play an important role in the spermatid maturation could be the reason of the scarcity of spermatids in the lumen. Finally the inhibition of the development of cytoplasmic organelles such as mitochondria could be an explanation to the reduction of sperm activity and life span released during mating events.

In the present study, it could be shown that, DDT and A54 could act synergically and cause disorders of the male zebrafish reproductive tract, reduction of the growth and changes in the spermiogenesis.

Bioakkumulation und östrogenische Effekte von DDT, Arochlor 1254 und ihre 1:1 Mischung auf Zebrafisch (*Brachydanio rerio*)

Zusammenfassung:

Akkumulationsraten, Eliminationsgeschwindigkeiten und Metabolisierungswege zahlreicher Chlorierter Kohlenwasserstoffe (u.a. DDT, PCBs) wurden in der letzten Jahren ausreichend aufgeklärt. Ihre Organismenspezifischen Wirkungen waren dagegen bis in die Gegenwart Gegenstand kontroverser Diskussionen. Das gilt auch für für mögliche endokrine Wirkungen, für Beeinflussungen der Spermiohistogenese und damit auch mögliche Veränderungen der Populationsvitalität. Durch eine kritische Literatur-Recherche lässt sich auch der Nachweis erbringen, dass insbesondere bei Stoffgemischen (u.a. PCBs) zwar die rückstandsanalytisch leichter nachweisbaren diskutiert, die ökowirksamsten jedoch mangels ausreichender Laborqualitätssicherungsstandards kaum berücksichtigt wurden.

Substanzen mit östrogener Wirkung sind ökosystemer naturgemäß von besonderem Interesse. Drei experimentell zu klärende Fragen stehen dabei im Mittelpunkt.

1. Besitzen die Chemikalien eine insbesondere männliche Geschlechtsorgane schädigende Wirkung?
2. Können bestimmte Chemikalien-Gemische männliche Östrogenrezeptoren (hER) binden und/oder aktivieren?
3. Sind bestimmte Entwicklungsstadien eines Organismus besonders empfindlich für spezifische Chemikalienwirkungen und können sie deshalb als Sreening-Test-Systeme eingesetz werden?

In vorliegender Studie wurde deshalb versucht, die Wirkungszusammenhänge von DDT und Arochlor 1254 (A 54) als Einzelsubstanzen und in 1:1-Gemischen auf östrogene Effektivität an dem Zebrafish (Brachydanio rerio) zu bestimmen.
Dazu wurden zunächst in Vorversuchen unterschiedliche Konzentrationen und Gemische der Chlorierten Kohlenwasserstoffe in Kurz- und Langzeit-Expositionstests auf ihre Wirkung überprüft.
Die Konzentrationen der Mischungen lagen zwischen 0.05 µg/l und 500 µg/l und unterschieden sich voneinander um den Faktor 10. Dabei stelle sich heraus, dass Testkonzentrationen von 500µg/l für Zebrafishe zu toxish sind. Die LC50-Werte wurden für

DDT und die Chemikalienmischungen bestimmt. Die Auswertung aller Testkonzentrationen zeigte, dass die Mortalität bei den Chemikalienmischungen am höchsten war. Deshalb wurde überprüft, welche Menge der Chemikalien von der Zebrafischen absorviert wurde. Dazu wurde der „static fish test“ 305D nach der OECD-Richtlinie benutzt (Bioakkumulationstest). Nach den im Test ermittelten Daten wurden das Experiment mit vier Konzentrationen von DDT, A 54 sowie deren 1:1-Mischung, wiederum in jeweils um den Faktor 10 verstärkten Dosen zwischen 0.05 µg/l bis 50 µg/l durchgeführt. Der achttägige Test der Bioakkumulation mit DDT und A54 zeigte, dass die Zebrafishe die Chemikalien aufnahmen und akkumulierten, doch stellte sich in allen Fällen kein Gleichgewicht ein. Eine Konzentration von 0.05 µg/l ertrugen die Zebrafische vor Ablauf der acht Tage; später waren die Chemikalien nicht mehr nachweisbar (NOEC-Wert). Bei allen anderen Konzentrationen stieg der Faktor der Bioakkumulation innerhalb der acht Tage (BCF8days) mit den jeweiligen Testkonzentrationen an. Die Werte des BCF8days-Tests belegten, dass Zebrafische DDT stärker als A 54 absorbierten.

Aufbauend auf diesen Analysen erfolgte die Untersuchung des Lebenszyklus der Zebrafische. Zunächst wurden die Eier verschiedenen Testkonzentratione der beiden Chemikalien und deren Mischungen ausgesetzt. Es stellte sich heraus, dass die Chemikalien signifikante Veränderungen der Brutzeit, der Pfortpflanzung und der Länge der Jungfische verursachten. Diese drei Parameter waren stramm mit dem Gehalt an Chemikalien korreliert. In allen Fällen überlebte kein Männchen eine Konzentration von 50 µg/l . Darüber hinaus ergaben sich Abweichungen zwischen der zu erwartenden und der experimentell ermittelten Länge ihrer Entwicklungsstadien, die je nach Pestizidgehalt um wenige bis viele Tage verzögert waren. Die bis zu sechswöchige Untersuchung der Entwicklungsstadien unter Einwirkung der Chemikalienmischung länger dauerte als bei reiner Pestizid-Applikation und dass die Zebrafische mehr A 54 als DDT vertrugen.

Während dieser Untersuchung wurde das Längenwachstum bestimmter Testexemplare gemessen. Mittels ANOVA lagen die F-Quotienten bei einem Freiheitsgrad von 4,4 bei 1,37, 1,65, und 0,88 für jeweils DDT, A 54 und Ihre Mischung. Das Längenwachstum der Zebrafische wurde bei höheren Testkonzentrationen stärker gehemmt und tendierte dazu, noch deutlich auszufallen, wenn sie in Testkammern aufwuchsen, die mit Chemikalienmischungen kontaminiert waren. Dass die Brutrate und der Anteil überlebender, erwachsener Männchen zurückgingen, sich die Entwicklungsstadien im Lebenszyklus verzögerten,

messbar waren und deshalb als „Konzentration unterhalb der Messschwelle“ (NOEC) einzustufen sind. Bei allen anderen Konzentrationen stieg der Faktor der Bioakkumulation innerhalb der 8 Tagen (BCF8days) mit der jeweiligen Testkonzentrationen in beiden Fällen an. Die Werte des BCF8days-Testes erwiesen, dass in dem vorgegebenen feuchten Milieu die zebrafischen DDT stärker als A54 absorbierten.

Dieser Studie folgte die Untersuchung des Lebenszyklus der zebrafischen; als Einstieg setze ich deren Eier verschiedenen Testkonzentrationen der beiden Chemikalien und deren Mischung aus. Es stellte sich heraus, dass die Chemikalien signifikante Variationen in ihrer Brutzeit, der Fortpflanzung und der Länge der Jungfischen verursachen. Diese drei Parameter waren eng mit dem Gehalt an Chemikalien verknüpft; die Fischen sich also gegenüber höheren Konzentrationen empfindlicher zeigten. In allen Fällen überlebte kein Männchen eine Konzentration von 50µg/l. Auch gab es Abweichungen zwischen der gewöhnlich zu erwartenden und der experimentell ermittelten Länge ihrer Entwicklungsstadien, die je nach Pestizidgehalt um wenige bis viele Tage verzögert waren. Die bis zu 6wöchige Untersuchung der Entwicklungsstadien der F-II Generation wies auf, dass die Ausbildung der verschiedenen Entwicklungsstadien unter Einwirkung der Chemikalienmischung länger dauerte als wenn die Pestizide pur verabreicht wurden und die zebrafischen mehr A54 als DDT vertrugen. Während dieser Untersuchung wurde das Längenwachstum bestimmter Testexemplare gemessen. Zum Vergleich der Ergebnisse lagen mit Hilfe der Fehlerrechnung (ANOVA) die F-Quotienten bei einem Freiheitsgrad von (4,4) bei 1.37, 1.65, und 0.88 für jeweils DDT, A54 und ihre Mischung. Das Längenwachstum der zebrafischen wurde bei höheren Testkonzentrationen stärker gehemmt und tendierte dazu, noch deutlicher auszufallen, wenn sie in Testkammern aufwuchsen, die mit der Chemikalienmischung kontaminiert waren.

Daß die Brutrate und der Anteil überlebender, erwachsener Männchen zurückgingen, sich die Entwicklungsstadien im Lebenszyklus verzögerten, die Population abnahm und das Wachstum der Testorganismen durch die Chemikalien und deren Mischung gehemmt wurden, könnte möglicherweise der Erkrankung der männlichen Geschlechtsorgane zurückgeführt werden. Deshalb wurden die Qualität und Quantität der Spermien, die die mit den Chemikalien kontaminierten männlichen zebrafishe während der Paarung ausschieden, innerhalb gewisser Zeiträume untersucht. Nach den Methoden von Leong (1988) und Shapiro et al (1994) wurde jeweils nach 24 Stunden, 2 Wochen, einem bzw. zwei Monaten eine Probe entnommen. Sie zeigte, dass die Abnahme der gemessenen Spermienzahl mit der Chemikalienexposition zunahm, Konzentration der Testchemikalien und Einwirkzeit

bestimmen danach Anzahl, Aktivität und Lebensdauer der Spermien. Diese Abnahme steigt bei Pestizid-Gemischen signifikant an.

Um mehr über die Ursachen der Spermien-Degeneration herauszufinden, wurden Untersuchungen zur Hodenstruktur und Spermatogenese durchgeführt. Hierzu wurden Schnitte des Hodengewebes in EPON-812-Kunstharz eingeschlossen und halbdünne bzw. extrem dünne Schichten herausgeschnitten. Diese wurden licht bzw. elektronenmikroskopisch untersucht.

Unter dem Lichtmikroskop ließ sich eine Verminderung des Spermatidengehaltes im Lobulärlumen der Hoden deutlich erkennen. Diese Verminderung des Spermagehaltes fiel umso stärker aus, je höher die Testkonzentrationen und die Einwirkzeit waren. Ebenfalls schrumpften unter Einwirkung der Chemikalien der deferente Kanal, was den Raum zwischen den Lobulärlumen weitete und bei höheren Konzentrationen noch deutlicher abgrenzte und vergrößerte. Auf diesem Stand der Untersuchung wurde der Grund für die Abnahme der Spermatogenese elektonenmikroskopisch vertieft. Dadurch konnte gezeigt werden, dass noch nach zwei monate Einwirkzeit der Chemikalien auf der letzten Stufe der Spermatogenese hauptsächlich als primäre Spermatiden vorlagen, dagegen aber nur wenige und manchmal sogar keinerlei sekundäre oder tertiäre Spermatiden, was die Anzahl der Mitochondrien reduzierte. Weiterhin wurde entdeckt, dass es keine heterophagen Vakuolen gab, deren Gegenwart im Zytoplasma ein Anzeichen phagozytischer Aktivität gegen Keimbestandteile wäre.

Allgemein könnte das Fehlen phagozytischer Aktivität, die eine wichtige Rolle in der Reifung der Spermatiden spielt, der Grund des Mangels an Spermatiden im Lumen sein. Schließlich mag sich also die Abnahme der Aktivität und Lebensspanne der Spermien, die bei den oben genannten Paarungen abgesondert wurden, dadurch erklären, dass die Entwicklung der Zytoplasma-Organzellen, wie beispielweise die Mitochondrien, gehemmt ist.

In der vorliegenden Studie konnte gezeigt werden, dass DDT und A 54 synergetisch wirken können und Krankheiten an den Geschlechtsorganen männlicher Zebrafische, Reduktion ihres Wachstums und nachteilige Veränderungen in der Spermatogenese verursachen.

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1) Introduction and literature review.

1.1) Environmental effects of the pesticides

In 1939, the organochlorine insecticides like DDT, lindane, aldrin, dieldrin, endosulfan and heptachlor were discovered and made available as commercial insecticides (Nagel, 1995; Oerke et al, 1994; Osborne, 1985; Ware, 1986). The best known insecticide of this group is DDT. It has been used since the 1940's for vector control (tsetsefly and mosquitoes), agricultural pests (Desert locusts, Army worms and other crop pests) and forest pests (Gypsy moth) (Jordan, 1986; Nagel, 1995; Oerke et al, 1994). The organochlorine insecticides are not easily broken down in the environment and persist for a long time. They are neurotoxic by blocking the process of synaptic transmission in the nerve system of vertebrate and invertebrate organisms.

The introduction of the organochlorine insecticides was followed soon by the development of organophosphate insecticides like parathion, malathion and fenitrothion, which are phosphoric esters produced synthetically and powerful inhibitors of acetylcholinesterase enzymes ie deactivating enzymes of the mediator (acetylcholine) of nerve transmission. The cerebral damage of these chemicals is irreversible. World wide, more than 75 of these compounds are in use as fast acting contact insecticides (Lund, 1985; Oerke et al, 1994; Osborne, 1985; Ware, 1986). Further research of new active ingredients resulted in the development of carbamates like sevin, baygon, aldicarb and carbofuran. These insecticides have systematic properties and can be used against soil pests (e.g. nematodes) as well as insects which are resistant to organochlorine and organophosphate compounds.

They tend to be rather more persistent than organophosphate in soil, but are less toxic. The carbamate insecticides behave like inhibitors in competition with the cholinesterases by settling on the active sites of the enzyme, but this damage is reversible (Oerke et al, 1994; Ramade, 1987; Ware, 1986).

The search for new alternative active ingredients was essentially intensified in the 1960's and 1970's. Additional compounds such as pyrethroid, for example deltamethrin, permethrin, cypermethrin, cyfluthrin and flumethrin were brought into the market in 1976. Pyrethriods are generally highly toxic to insects but less toxic to mammals and they do not accumulate in the environment (Oerke et al, 1994; Nagel, 1995; Ruigt, 1985; Ware, 1986).

The detection of the adverse effects of the neurotoxic compounds used for combating pest arthropods in the 1960's led many industrialised countries to change their way of pest control

programmes and induced the search for alternative insecticides which are safe both to human beings and the environment. Entomological research was among others directed to modifiers of insect behaviour, metabolic disrupters and moult inhibitors such as insect growth regulators (IGRs).

Chemicals used to control pests are most often non specific in action ie, they do not only affect target organisms but also kill a number of other beneficial insects like predators, parasitoids and pollinators or neutral species (non target) and hence significantly disturb their ecological role in the natural ecosystem. These chemicals are either synthesised (xenobiotics) or derived from natural occuring plants. Currently, approved active ingredients used in pesticides amounts to approximately 700 worldwide and their ecotoxicological side effects in particular on beneficial non target organisms are sometimes not well documented despite the testing requirements for registration. The number of different formulations registered for commercial use is even higher and was estimated at 10,000 (Römbke & Moltmann, 1996; Ware, 1986). There is insufficient database of the chemicals used today, eventhough there are a large number of ecotoxicological standard methods to assess the toxicity and behavoiur of these compounds in the environment.

Ecological impacts of conventional insecticides in connection to control insect pests like trypanosomes which are affected and transmitted as the tsetsefly of the genus (*Glossina* spp, Glossinidae: Diptera), desert locust (*Schistocerca gregaria*, Aricidae: Orthroptera) which are affected 57 countries of Africa, Asia and Europe, nuisance insects like midges (*Chirononus* spp) gypsy moth (*Lymantria* spp) forest pest in Europe and North America, pest ants been documented in many parts of the world.

Organochlorine insecticides are still being used and even produced in some developing countries like India, eventhough the production and use of DDT and related compounds such as lindane and dieldrin has been banned since the 1970's in the industrialised world, mainly Europe and North America (Ramade, 1987; Van der Valk et al, 1988). The ecological damage of these chemicals has been continuosly occuring in many parts of the earth's surface nearly 40 years after the evidence has shown up that organochlorines accumulate in food chain and different trophic levels in the clear lake California, which had been treated several times with DDT (Crane et al, 1983; Everts et al, 1985; Koeman et al, 1971; Müller, 1988; Nagel, 1995; NRCC, 1981; Peveling et al, 1994; Van der Valk et al, 1988). It is also observed both types of effects, direct effect, resulting the death of organism and indirect effect manifested the reproduction of organism and disturb food chain or key species which play important role in the structure and function of the ecosystem. Side effects of pesticides have been also observed

on individual, population, community, and ecosystem level. The severity of the impact of insecticides to the non target organisms depends on the application method, dosage, formulation of the pesticide, amount of land covered, and the sequence of treatments (Atkins et al, 1986; Croft et al, 1982; Everts et al, 1989; Grant, 1989; Keith, 1992):

The toxicity study of the chemical in the laboratory using selected species are at best but have limited value for predicting chronic effects of chemical in situ. Extrapolation of data obtained from single species and single process tests to higher levels of ecological structure and function (community, ecosystem) make the risk assessment and decision making an even more challenging task. However, several ecotoxicological studies on organochlorines, organophosphates, carbamates, pyrethriods and insect growth regulators reveal that these substances can disturb biological diversity and ecological integrity , ie the sum of the variety of the earth natural occuring elements and processes. Degradation of integrity leads to a biotic impoverishment of the earth (Müller, 1988; Karr, 1993; Pascoe, 1993).

Organochlorine compounds (OC's), synthesised as pesticides and for industrial purposes, have been in use since the 1940's. With the growing evidence of negative persistent environmental effects, the use of these xenobiotics was considerably curtailed by the late 1970's in most industrialised nations. But DDT for example is still widely used in tropical countries for pest and malaria control and approximately half of the world production of polychlorinated biphenyls has not yet escaped to the environment from older electrical and other equipment. The chemically stable nature of most of these compounds, combined with their resistance to biodegradation and photolysis, has resulted beside the direct contamination in industrial areas in a global distillation via the atmosphere (Ballschmiter, 1992). The Wadden sea is influenced, beside atmospheric input, mainly through the effluxes of the great rivers Rhine, Weser and Elbe and through a constant exchange with North Sea waters (CWSS, 1991).

OC distribution in the aquatic environment can be understood by considering the distinctive physiochemical properties of low water but high lipid solubility and relative inertness of these compounds. In its simplest form, an equilibrum is hypothesised to develop between concentrations in water and lipid phase of marine organisms (Falkner & Simonis, 1982; Kawamoto & Urano, 1989; Bierman, 1990; Pruell et al, 1993). Rather than a purely physical partitioning between these phases, organochlorines are thought to absorb, in an intermediate step, onto the surface of all particulate material (Harding, 1986; Opperhuisen and Stokkel, 1988). In the case of aquatic invertabrate organisms, OC's are then observed through the cell wall and transferred to lipid rich organelles or tissues. Residues of OC pesticides and

industrial compounds such as polychlorinated biphenyls(PCB's) are continuosly monitored in fish from the North Sea and other costal areas. Studies of the variation in contamination level in relation to geographical distribution are based mainly on the analysis of pooled fish species, such as atlantic cod, whiting, plaice, dab and herring. The results provide useful information on the pollution of the inshore marine environment and temporary trends (North Sea Task Force, 1993).

Information on the contamination level of fish in relation to spatial distribution (eg fish ground) and biological conditions (seasonal variation of the fat content, accumulation by age or length) are rare (Kamman et al 1990; Miller, 1994). Most recently, Westernhagen et al (1995), reported on the content of chlorinated compounds in the ovaries and livers of various fish species and found, in general, no correlation between accumulation and age or size. Kruse and Krüger (1989) reported on the contamination level of various fish species from different fishing areas, showing that the levels of organochlorine pesticides and PCB's were well below the limits laid down in German regulations and directives (Bundesminister für Gesundheit 1988, 1996). Kelly and Campbell (1994) compared the concentrations of organochlorine residues in herring muscle tissue from three locations around Scottland.

1.2) Estrogenic properties of DDT and PCB´s

Early studies in mammals and birds with chlorinated hydrocarbons (CH's) of the DDT class demonstrated that these compounds exhibit effects which could be interpreted as causing alterations of the hormonal state of the animal. In turn these effects by CH's appeared somewhat conflicting and could be attributed to either estrogenic, antiestrogenic or antiandrogenic actions (Kupfer. D, 1975).

The probable reasons for the somewhat confusing observations were due to several factors: (a) studies were conducted with technical grade DDT preparations containing several DDT isomers; the p,p' isomer is the major component of technical grade DDT, however the o,p' isomer is usually present as a major contaminant (up to 20%); (b) p,p' DDT is a potent inducer of the hepatic microsomal cytochrome p-450 monooxygenase (Hart & Fouts, 1963; Juchau et al, 1966) which catalyzes the metabolism of numerous xenobiotics and steroids, among these estrogens and androgens. Hence enhanced metabolism of an estrogen or androgen (endogenous or administered) by pretreatment with p,p' DDT would probably result in a decrease in estrogen and androgen activity and likely would be interpreted as an antiestrogenic or antiandrogenic action of DDT.

The chlorinated hydrocarbon pesticides related to DDT are only active as estrogens in milligram amounts, a 1000-fold difference. The DDT analogs are not phenolic, but they may give rise to aromatic phenolic substitution during metabolic conversions in the animal. DDT analogs are compounds of the diphenylethane type. Other analogs are diphenylmethane or triphenylmethane series. Polychlorinated triphenyls have been examined and found to be compounds which have become increasingly implicated as environmental pollutants of industrial origin.

In a series of polychlorinated biphenyls, the compounds containing up to 48% chlorine were active when the 18hrs glycogen response of the immature rat uterus was used.

In the 1950's, it has been already theorized that a rather large, rigid lipoid soluble molecular structure with two active hydrogen-bound forming groups located at an optimum distance of 14,5Å units from each other would be estrogenic. They further stated that potency is decreased as the distance between the groups is decreased or increased while DDT possesses a relatively large, rigid, lipoid soluble molecular constitution, it does not present active hydrogen atoms at the hypothesized optimum distance of 14.5Å. However the presence of electronegative chlorine atoms in the p,p'-orientations would prohibit the existence of active hydrogen. If these p,p' chlorine atoms were metabolized to group prossessing active hydrogen, the possibility of estrogen action would exist. The general lack of estrogenic activity of p,p'-DDT analogs suggests that such metabolism doesn't occur readily in the biological situations studied thus far.

Conversely, the activity of o,p'-DDT raises interesting theoretical relationships between chemical constitution and estrogenic activity. The o,p' chlorine atoms are not at the hypothesized optimum distance. The exact nature of the active estrogen structure arising from o,p'-DDT, it is not o,p'-DDT itself, might provide important information relating to the spatial configuration of an active estrogen.

The structural observations regarding estrogenic activity in DDT and PCB's compounds indicated that activity is conferred when a p- or p'- position is inoccupied (-H), or substituted by –OH or OCH_3. Some polychlorinated biphenyl compounds exhibit estrogenic activity. Measurements of internuclear distances of Dreiding steric models indicated that active sites would be 9-11Å apart a range similar to those found in natural and synthetic estrogens. The PCB's have been reported to be uterotropic in rats. These compounds have been widely used as sealants and additives to paints, plastics, rubber, adhesives, printing inc and insecticides. These compounds represent important environmental contaminants and as such have been found in human milk. Gellert in 1978 has reported a correlation between the weak

estrogenecity of some PCB's mixtures (respectively Arochlor 1221) and their ability to permanently alter the pattern of neuroendocrine reproductive function in rats exposed during development. However PCB's have been reported to reach the fetus after the maternal exposure in a number of species including humans (Akiyama et al, 1975).
Thousand of chemicals are introduced in our environment with little knowledge of their effects on two physiological processes which are central to our survival as a species…, reproduction and development. With millions of women taking oral contraceptives, some environmental contamination with estrogenic materials is a distinct possibility.

1.3) Fish and environmental chemicals

Fish as a group are of great importance for man. Even today fish remain a major source of protein for many peoples, and in the industrialized nations, commercial fisheries and fish processing industries represent important sectors of economy. Apart from the significance of fish as a direct food source for man, processed fish meal plays a prominent indirect role in human nutrition as an important feed in the production of meat.
In an attempt to evaluate the effects of environmental chemicals on fish, it is essential to recognize that "fish" as archetype does not exist. Bony fish in terms of zoological taxon (Osteichthyes) comprise the vast majority of all fish and among these, teleost represent the dominant recent subclass. Estimation of teleost diversity range between 20,000 and 30,000 species (Altman and Dittmer, 1972), and structural and biological diversities of fish are immense. In almost any limnic and marine habitat, a great variety of different ecological niches has been occupied by teleosts, and as primary, secondary, and tertiary consumers, they can be placed in several trophic levels within aquatic food nets. It is essential to take this considerable morphological, physiological, and ecological diversity of fish into account during investigations of potential interactions between environmental chemicals and fish.
For an adequate evaluation of potential hazards of chemicals, the knowledge of whether a substance can be resorbed, and if so, at which amount a substance is taken up by fish is an indispensable precondition. Uptake may occur directly from the surrounding water or via food and, itself does not yet represent damage. Harmful effects can only be induced, when the substance has been accumulated in sufficiently high concentrations at the site of toxic action. For instance to be deleterious, genotoxic agent must come into contact with chromosomes. Since distribution, metabolism, storages and excretion profoundly affect the concentration of xenobiotics at their site of action, these processes must be taken into consideration.

After uptake by an organism, the amount of a xenobiotic compound which cannot be excreted, will persit in the body and will be accumulated. Thus, bioaccumulation has been defined as the process of concentration of xenobiotics within the body, no matter whether these are directly absorbed from the surrounding medium, i.e, in the case of fish from the surrounding water, biomagnification means the accumulation of xenobiotics from food within the food chain or web.

In the assessment of potential hazards of chemicals in aquatic systems, the accumulation of chemicals within an organism is determined as bioconcentration factor (BCF) using as a rule, fish as test organisms. For the investigation of BCFs, ther are several different guidelines set by the Organization for Economic Cooperation and Development (OECD, guidelines 305, parts A-E), and numerous investigations using a variety of fish species have demonstrated that there is a good correlation between the bioconcentration of environmental chemicals in fish and the substance-specific octanol/water partition coefficient logPOw as a measure for the lipophilicity of the compound. The correlation between logPOw and bioconcentration is roughly linear up to logPOw values of 6-7, indicating elevated accumulation with increasing lipophilicity of the substance.

The evaluation of bioaccumulation as a factor of toxicity has been contrasted by some authors. For instance according to McKim and Schneider (1991), bioaccumulation is not reflecting toxicity. This statement is based on the relationship between logPOw higher than 2.7 and 3.0, which are the trigger values within the chemicals and Plant Protection Acts of the Federal Republic of Germany, respectively (Beek, 1991). Once accumulated in an organism, Xenobiotic compouds may cause biochemical, physiological, morphological, and genetic alterations in fish. These alterations can influence specific performances of the organisms, e.g. development, growth and reproduction.

The effects may be sublethal or may result in the death of the animals. In the chemical Act, the test scheme for the estimation of chemical toxicity comprises three stages according to the reproduction rate of the chemical.

Base level: fish test in the acute toxicity range (LC50 (96h))

Level 1: prolonged fish test (14 and 28 days, respectively).

Level 2: long term studies including reproduction tests.

There is no such rigid scheme in the Plant Protection Act; yet the acute fish test is also the initial step in the procedure. The impact of the chemicals on early life stages (ELS) of fish may, e.g be tested according to the OECD guidelines 210. A ring test including eight laboratories testing 3,4- dichloroaniline effects on zebrafish has documented an ELS test with

fathead minnow (*Pimephales promelas*, call et al , 1987) and results show that zebrafish is apparently slightly sensitive than the fathead minnow. In a comparative study of 3,4-dichloroaniline effects on early life stages of zebrafish, perch *(Perca fluviatis*), and roach (*Rutilus rutilus*), Schäfers (1991), the three different species displayed similar susceptibility.
Long term test should include investigations of the effects of environmental chemicals on the reproduction of fish. In the laboratory, this can be achieved by conducting either an abbreviated or complete life-cycle test.. Where as abbreviated test is restricted to the measurement of egg production followed by an early life stage test, the complete life cycle test comprises effects of permanent exposure to the test substance on embryos, larvae during embryo larval development in the F-II generation.
As a matter of course, within a reasonable period of time, complete life cycle tests can only be carried out with small rapidly growing warmwater fish such as the zebrafish (Brachydanio rerio), an egg laying species, which reaches sexual maturity only after three months.

1.4) Toxicity of DDT and PCB's to fish

1.4.1) Toxicity of DDT to fish

There is a number of reports on the relationship between sublethal treatment with chlorinated hydrocarbon insecticides and behavioural changes in fish, for instance J.M. Anderson and Peterson (1969) studied how treatment with low DDT concentrations (20-60 ppb for 24hours) affected behaviour directed by the central nervous system (CNS). They found the temperature at which a simple (propellar tail) reflex was blocked to be higher for treated brooktrout; ie treated fish has less thermal acclimation ability.
According to Meyer and Ellersieck (1986), DDT is toxic to fish; the 96 hours LC50 reported (static tests) range from 1.5 to 56µg/l for large mouth bass and guppy respectively.
Smaller fish are more susceptible than larger ones of the same species. The uptake of DDT from water is affected by the size of the fish; smaller fish take up relatively more DDT from water than larger specimens of the same species in a particular given area of the culture. For instance, Murphy (1971) reported that a range in weight of mosquitofish between 70 and 1000mg led to a four-fold difference between the smallest and largest fish in DDT uptake from water over 48h.
The exact mode of action of DDT in fish remains unclear. There have been many different suggestions to explain both lethal and sublethal effects. Most of these are primarily the result

of the effects on membranes. DDT may interfere with membrane function and with many enzyme systems that are located on membranes. It has been shown experimentally to interfere with the normal function of so many systems that a primary action of DDT is difficult to determine. Death as an endpoint represents an unambiguous parameter for the fish. However, unless the normal effects include large parts of the population (as eg during massive fish kill after the chemical spill into the Rhine river at Basel in november 1986), the significance of the death of individuals for the population is unclear, since evaluation and prediction of chronic effects on the basis of acute toxicity data is in principle, impossible.

1.4.2) Toxicity of PCB's to fish

Fish of all life stages have been shown to take up PCB's readily from water; bioconcentration factors are high. Time taken to reach equilibrum is variable, but often long, in excess of 100 days. PCB's with greater chlorination are more readily taken up and retained. PCB body burden tends to increase with age and levels are higher in fish with a greater lipid content. The accumulated DDT are concentrated in lipid rich tissues. PCB's of lower chlorination are eliminated more rapidly. Loss of PCB's is evident when exposure ends; an initial rapid loss is followed by a slower rate of loss. Half life estimates, therefore vary greatly, from a few weeks to several years. Reproduction with the production of large mass of eggs or sperm, allows loss of substancial amounts of the PCB residue. Depending on the species, habitat, and behaviour, PCB'S can be taken up from water, sediment, or food to different degrees.

As an example, DeFoe et al (1978) exposed fathead minnow (*Pimephales promelas*) to Arochlor 1248 or 1260 at concentrations of 0.1-3µg/l, for 240 days (life cycle). Bioconcentration factors for the uptake of PCB's were independant of the PCB's concentrations in the water. Residues in the fish reached an apparent steady state within about 100 days of exposure and growth. Females accumulated about twice as much PCB's as males, because of their higher body lipid content. Although mechanisms for uptake were similar for both Arochlors, greater body burdens were always achieved with exposure to Arochlor 1260. Bioconcentration factors ranged from 60,000 to 160,000 for males and from 120,000 to 270,000 for females. After transfer to clear water, 18% of Arochlor 1248 was lost within 28 days ad 15% of Arochlor 1260 in 42 days. The authors stated that, because of variations between fish, this 10-20% decline in total body burden of PCB's was insufficient to indicate definite PCB elimination over this period.

1.7) Statement of the problem and justification of the study

Increasing environmental concern and man's eagerness to protect his health and environment made many countries shift from conventional insecticides to biological control agents environmentally safer chemicals. Today emission is in the form of gas and dust, radioactive substances, pesticides and wise effect human beings, animals and plants living in the biosphere. Large quantities of anthropogenic chemicals released into the environment provoke umpredictable changes on a short and long term basis.

DDT and PCB's are not easily broken down in the environment and persist for a long time and can also kill a number of beneficial neutral species (non target) and hence significantly disturb their ecological role in the ecosystem. The ecological damage of these chemicals has been continuosly occuring in many parts of the earth's surface nearly 40 years after the first evidence has shown up organochlorines in food chain at different trophic levels in the clear lake California which have been treated several times with DDT.

Persistence and bioaccumulation of residues of organochlorine pesticides in foods of animal origin has been established world-wide.

Some estrogenic activities of the chemicals have been studied and found to be of great ecotoxicological importance, since they could influence the reproduction, which in turn could provoke an impact on the population and the ecosystem.

It has been interpretated that the environmental estrogens could increase the incidence of human breast and testicular cancers and decreasing male sperm counts and sperm quality (Arnold et al, 1996).

Today concerning estrogens in the environment, researches are mostly based on the differentiated generations and biological reproduction in wild life, for instance in fishes, reptiles and birds; the results of research with fishes like bioindicators in flowing domain of purification and finally on the new epidemiological investigations for the functional development of several organs in human beings especially in men.

In 1993, Sharpe and Skakkebaek were still searching to know whether estrogens are involved in falling sperm counts or cause disorders of the male reproductive tract.

On the other hand, referring to the fish toxicity tests, there are still some contrasting reports on the concepts of ELS and complete life cycle (CLC). Most recently, Nagel (1994) hoped that CLC tests could be shown to be useful tools in fish toxicology.

In the present study, I first of all investigated the short and long term exposure of the zebrafish to DDT and Arochlor 1254 in order to evaluate the rate of mortality and the LC50

after a certain time. I further performed the bioaccumulation test to know the exact amount of the chemical picked up by the test organisms at the beginning of the experiment. This point led me to proceed the experiment by investigating the effect of the chemicals on the duration of the life cycle stages (LCS), the reproduction, and the growth of fish up to early stage of the F-II generation. These investigations could help me to estimate how far was the effect of the chemicals widespread within the stages of the life cycle, and how sensitive was the growth of juvenile fish emerged to the chemicals as well as all the reproductive disorders that the latters could cause.

The specific objectives of this study were:

(a) To know which amount of the chemicals are taken up by the zebrafish by evaluating the bioconcentration factors (BCFs) at each of the tested concentrations

(b)To evaluate the effects of DDT, Arochlor 1254 and their 1:1 mixture on the mechanism of sperm release in Zebrafish in order to establish how the semen volume variates.

(c)To investigate the ultrastructure of the testis of male zebrafish exposed to the chemicals and their 1:1 mixture which could help to bring out the impacts observed on the spermiogenesis and to discuss about the quality of the sperm produced .

2) Materials and Methods

2.1) Short and long term exposure

2.1.1) Experimental water in test chambers

The water used during the experimental period was reconstituted water ISO 6341-1982 according to the OECD guidelines for testing of chemicals 203. It was prepared as follows:

calcium chloride solution

dissolve 11.76g $CaCl_2.2H_2O$ in deionised water, make up to 1 litre with deionised water.

- Magnesium sulphate solution

dissolve 4.93g $MgSO_4.7H_2O$ in deionised water; make up to 1 litre with deionised water.

Sodium bicarbonate solution

Dissolve 2.59g $NaHCO_3$ in deionised water; make up to 1litre with deionised water.

- Potassium chloride solution

dissolve 0.23g KCl in deionized water ; make up to 1 liter with deionized water.

The conductivity of the distilled or deionised water should not exeed $10\mu Scm^{-1}$.

25 ml of each of the 4 solutions above mentioned, were mixed and the total volume made up to 1 litre with deionised water.

The sum of calcium and magnesium ions in this solutions was 2.5 mmol / l. The dilution water was aerated until oxygen saturation was achieved, then stored for about two days without further aeration before use.

2.1.2) Experimental procedure

70 zebrafish (*B.rerio*) aged 12 weeks approximately, weighing between 0.54 - 0.6g and measuring 3 to 3.5cm were used in this part. They were supplied by a zoological store in Saarbrücken, Germany. They were kept in 50 litres glass aquaria filled with tap water in the laboratory and allowed to acclimatize for a period of 14 days.

Afterwards, 10 litres aquarium were filled with reconstituted water, with a ph of 8 and a dissolved oxygen concentration of 90% of air saturation. The temperature was regulated at 25°c in each aquarium.

10 fish were transferred in each of the 10 l aquaria and were allowed to stay 8 days in a static condition. The fish were then fed with Tetramin ad libitum and the feeding was stopped 24 hours before the exposure to the different tested concentrations of the pesticides commences.
On the day 8, the tested concentrations of the pesticides and their mixtures were prepared. DDT and Arochlor 1254 were then solubilized in ethanol at the rate of 0.1 ml / l and the tested levels were 0.05, 0.5, 5, 50 and 500µg/l for each chemical as well as their 1:1 mixture. Each of the prepared tested levels were then applied to the 10 liter aquaria containing fish, and only one of the aquaria was treated with 0.1ml of ethanol, which then served as control.
After 96 hours of exposure to the chemicals, the condition was transformed into a semi-static condition with the batchwise renewal of water in test chambers each 48 hours. The water hardness, the dissolved oxygen, the ph and the temperature were regulated and maintained in each aquarium as it was the case in the static condition.
In each situation, the observation started three hours after exposure to the chemicals and mortality recorded on a period of 96 hours for short term and 21 days for long term.
The evaluation of the LC50 was performed according to the Methods for calculating an LC50 of Stephan in 1977.

2.2) Bioaccumulation of the pesticides: static fish test

2.2.1) Description of the experiment

Zebrafish aged 3 to 4 months were first kept at a stocking density in glass aquaria for 2 weeks. Afterwards they were shared in 30 liters aquaria fiiled with reconstituted water ISO 6341-1982 and contaminated with 0.05, 0.5, 5, and 50µg/l of DDT and Arochlor1254 respectively. Alternatively, the other aquaria were also filled with reconstituted water contaminated with the same levels of the chemicals but without fish and were then called the blank medium.
For each chemical, 5 aquaria were placed, filled with 25 litres of medium, saturated with oxygen, at 24 ± 2°c. Provisions were made to supply them with compressed air to avoid oxygen concentrations < 5mg per litre.
Two aquaria were first dosed with two different levels of the test substance and the fish aquarium containing the blank medium. (Figure A) represented below.
Immediately after dosing and mixing, the water samples were taken from all aquaria.

The samples from the blank were used as a blank for the analytical procedure. (The medium being previously checked for contamination). The samples were analyzed for the test substance and delayed untill the following three samples of each aquarium have been taken.
Twenty fish randomly chosen from the stock were then added to the blank aquarium (biological blank) and to one of each of the two aquaria with the same concentration of test substance.
One aquarium with each level of test substance remained without fish (chemical blank).
The fish were fed at t = 2, 4, 6 days using 20mg dry food each gram fish with Tetramin after samples have been taken.
The samples of the medium from each aquarium containing the test substance were taken after 6, 24, 48, 96, 144, 192 hours, and after 192h (at the end of the test) also from the biological blank.
The concentration of the test substance was preferably analyzed within 48 hrs.
The oxygen concentration in all aquaria was measured at least daily, more frequently when it was critical (< 6mg per litre). The aquaria were then aerated (also those without fish) with approximately the same amount of air.
At the end of the test, the ph was measured in all aquaria.
The samples of 10 fish from each dosed aquarium were taken after 192 hrs. The fish were drained and wet weight of each quickly determined. Each sample of 10 fish was placed in a small aquarium all glass jar or other suitable uncontaminated container. They were homogenized and placed in a tighly closed container and keep deep frozen until analysis. A combined sample of 10 fish was made to minimise the effect of variation between individuals.
The fish in the blank aquarium were used to determine the average wet weight and fat content of the fish at the end of the test.

2.2.2) Application for water sample preparation based on Solid Phase Extraction (SPE)

2.2.2.1) DDT from water samples

The extraction of DDT from water samples was performed using a column type CHROMABOND® C18ec/ 6ml/ 500mg described as follows:
The samples were first filtered to remove big particles. The column was conditioned with 2 column volumes ethyl acetate, then 1 column volume methanol, and finally 1 column volume distilled water. The samples were then slowly pressed or sucked through the column.

Thereafter, the column was washed with distilled water and dried 15 min thoroughly under vacuum.
2 ×500ml of ethyl acetate were used for the elution.

2.2.2.2) Arochlor 1254 from water

The extraction of Arochlor 1254 from water samples was performed by using a column type CHROMABOND® C18/ 3ml/ 500mg described as follows:
500ml samples was mixed with 60ml 2-propanol, and the column was conditioned with 2 column volumes methanol, then 2 column volumes distilled water/ 2-propanol (85:15, v/v). The sample was then slowly pressed or sucked through the column which thereafter was washed with 2 ×750µl distilled water / 2-propanol (85:15 v/v).
Finally the elution was done by using 2 × 500µl of dichlormethane.

2.2.2) Analytical procedure

The eluat of water samples and the homogenized fish samples were applied to the Standard Operating Procedure (SOP). The SOP for analysis of pesticide residues in biological matrices according to the US-EPA method 608 (US-EPA, 1980) was used for all the fish samples with slight modifications in consideration of the differences in the fat contents. The extraction procedure was the same in all the fish samples, save for the amount of sample extracted (100-1000mg). The respective amount of sample ranging between the above mentioned values was weighed and ground in a porcelan mortar containing anhydrous sodium sulfate to yield a dry free-flowing powder. For the fish samples sea sand was also added to the mortar during grinding. Each sample was spiked with 20µl of a surrogate during extraction.
The dry free-flowing powder was transferred into a glass extraction column of a length 30cm and an internal diameter of 2cm. The dry column was then eluted with 80ml of dichlormethane with the first 40ml allowed to stay in contact with the powder for 20 or 30 minutes. The eluate was collected in a pre-weighed round bottom flask.
Dichlormethane in the eluate was removed using a rotatory evaporator at about 35°c and under reduced pressure of –0.5 bar. The flask was weighed until a constant weight was obtained, and the amount of extractable fat determined. The glass column was filled with about 10cm of distilled petroleum ether. Florisil ® (3% water) was added to the column depending on the amount of fat. Uniform packing of Florisil ® was ensured by gently tapping

the column. Below are the quantities of Florisil ® used for the different amounts of fat in the samples:

Fat (mg)	Amount of Florisil ® (mg) used
0- 200	10
201- 300	15
301- 400	20
401- 500	25

Water samples were first flitered to remove big particles and extracted with ethyl acetate according to the column type CHROMABOND® C18ec / 6ml/ 500mg for the estimation of DDT. These water samples were considered to have 0g fat and then ranged in the first category of samples during the clean up.

Both fish and water extracts at this level were redissolved in 2 to 5ml of petroleum ether and pipetted on the top of the column. The flask was rinsed twice with about 2ml of eluting mixture. This was followed by elution of the column using a mixture of petroleum ether / dichlormethane at the ratio of 4:1. The flow rate of the elution mixture was controlled so as not to exceed 5ml/ min.

The amount of elution mixture used were as follows:

Florisil® (g)	Elution mixture (ml) used
10	120
15	180
20	240
25	300

The eluate was concentrated to a small volume (approximately 5ml) using a rotatory evaporator at about 35°c under reduced pressure at –0.6 bar. This was then transferred to a 10ml flask and evaporated to a gently dryness. One milliliter of internal standard was added to the flask, mixed thoroughly with a whirl mixer and then transferred to autosampler vials ready for gas chromatography. For fish the end volume was reduced to 0.5ml.

A GC with the following parameters and operating conditions was used for the residue analysis:

GC instruments: PE Sigma 300 with AS-100 autosampler and "Spectacle" analysis system.

Detector: ECD

-Carrier gas: hydrogen (0.95 bar)

Make-up gas: Ar/CH_4, 20ml/min

Column : 30m FS- optima-5-MS-0.25 (Machercy- Nagel).

-Stationary phase: Optima- 5 (5% phenyl-, and 95% methylpolysiloxan)

internal diameter : 0.25mm.

film thickness: 0.25µm

-Temperatures: detector 300°c

injector 250°c

oven 90-250°c

Temperature program of oven:

Temp (°c)	Rate(°c/ min)	Time (min)
90	-	10
160	25	10
275	3	5

injection: 1µl splitless (1min)

Detection limit: 1p

Quantification of the residues was carried out using Spectacle® software program (LabControl, 1994). The detection limit varied from 0.0001 to 0.0005mg/kg fresh weight. A chromatogram of the standard DDT and Arochlor mixtures was used for quantification of residues. The levels of the pesticide residues in ng/µl obtained from the Spectacle® report were converted to mg/kg fat and fresh weight using Quanti 98 ® software program. The latter programme, trilored for the residue computation, was written by Dr. J. Krüger (Krüger, 1999, personal communication).

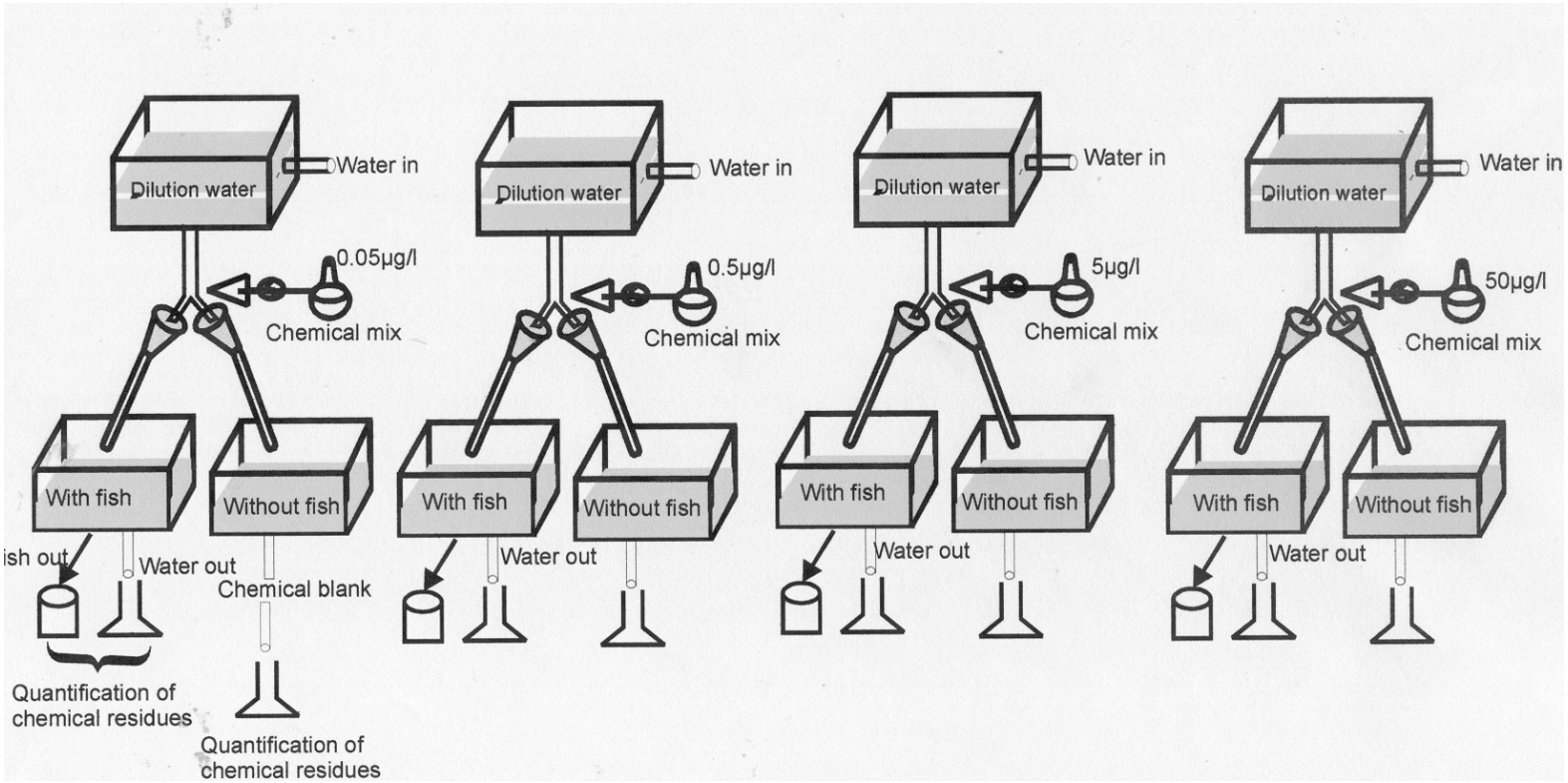


Figure A: Bioconcentration test within 8 days- static fish test

2.3) The tested chemicals

2.3.1) DDT

The chemical DDT was supplied by the company Promochem GmbH in Wesel, Germany.

Product identification:

120800 DDT (technical)

CA 1,1,1- Trichloro-2,2-bis (p-chlorophenyl)ethane(mixed isomers).

IUPAC mised isomers of o,p'-DDT(<30%) and p,p'-DDT

Formula C14H9C15

Mol. Weight 354.51

CAS No. 50-29-3

-physical data:

phase: waxy solid — vapour pressure 1.7E-7mbar at 20°c

Colour: colourless — solubility in water 1.2E-6g/l at 27°c

Melting point: 79.96°c — solubility in ethanol 500g/l at 27°c.

2.3.2) Arochlor 1254

The PCB was supplied by the company Merck in Germany and was made of the following commercial mixtures:

Arochlor[a]	clophen[b]	phenochlor[c]	Kanechlor[d]	Av.No.Cl/ Mol	Approx Wt.% Cl	Approx " Mol.wt"
1254	A50	DP5	500	5	52 – 54	326.4

a Monsanto Industrial Chemicals Company, USA

b Bayer, GFR

c caffaro, Italy

d Kanegafuchi chemical company, Japan

Source: Brinkman and Dekok, 1980. reproduced with permission, copyright 1980, Elsevier Biomedical Press BV.

2.3) Assessment of the life cycle stages and length of the experimental fish

In Europe zebrafish , (*B.rerio*) are increasingly used in toxicological studies. The fish well known to breeders measures 3-5 cm in the adult state. It thrives both in soft and hard waters, grows quickly at temperatures around 26°c and reaches the sexual maturity within 3 months. Zebrafish obtained from a commercial dealer and subsequently bred in the laboratory, were used as test specimens in the present investigation.

Mature females were mated with males in a glass aquarium with a breeding trap. Soon after spawning they were removed together with the breeding trap from the aquaria in which the eggs thus obtained were kept successively.

The aquaria were aerated constantly with a dissolved oxygen concentration of 9.1 ± 0.2 mg/l, regulated at 25 – 26°c under the natuaral day length with a light regime of 12 to 14 hours daily. The test began with fertilised eggs and followed the development of fish until their sexual maturity. After investigation of the reproduction and length, the test was continued with the development of fish in the F-II generation.

90 fertilised eggs were then contaminated with different DDT, Arochlor 1254 and their mixture concentrations of 0.05, 0.5, 5, 50 and 500µg/l solubilized in ethanol at the dose of 0.1ml/l in 3 replicates of 30 eggs each.

The water was not changed following hatching in order to minimize the disturbance for normal development of feeble fry, and maintained with temperature regulated and well aerated. Immediately after hatching, the test organisms were fed with Tetramin ad libitum a week later.

Eggs which showed a marked loss of translucency and change in colouration leading to a white opaque appearance, and larvae and juvenile fish which lacked reactions to mechanical stimulus were assumed died and removed from the test chambers.

The length of fish at each of the tested concentrations where they emerged was then evaluated on fixed specimens.

2.4) Mechanism of sperm release

Mature males and females zebrafish were maintained sexes separated in large aquaria and fed with Tetramin. The males were thereafter exposed to tested levels of DDT, Arochlor 1254 and

their 1:1 mixture within a period of 2 months. The sperm counts were performed 24 hrs, 2 weeks, 1 month and 2 months after exposure.

Before the collection of the samples, 2 males of each treated aquarium were introduced into a smaller aquarium (30L) and allowed to acclimatize for 3 days. After introduction of a ripe female, additional 80ml water samples were removed from the surface of the water with a beaker. (Figure B)

All the water samples collected were analysed for the presence of sperm using the methods of Leong (1988) and Shapiro et al (1994), described as follows:

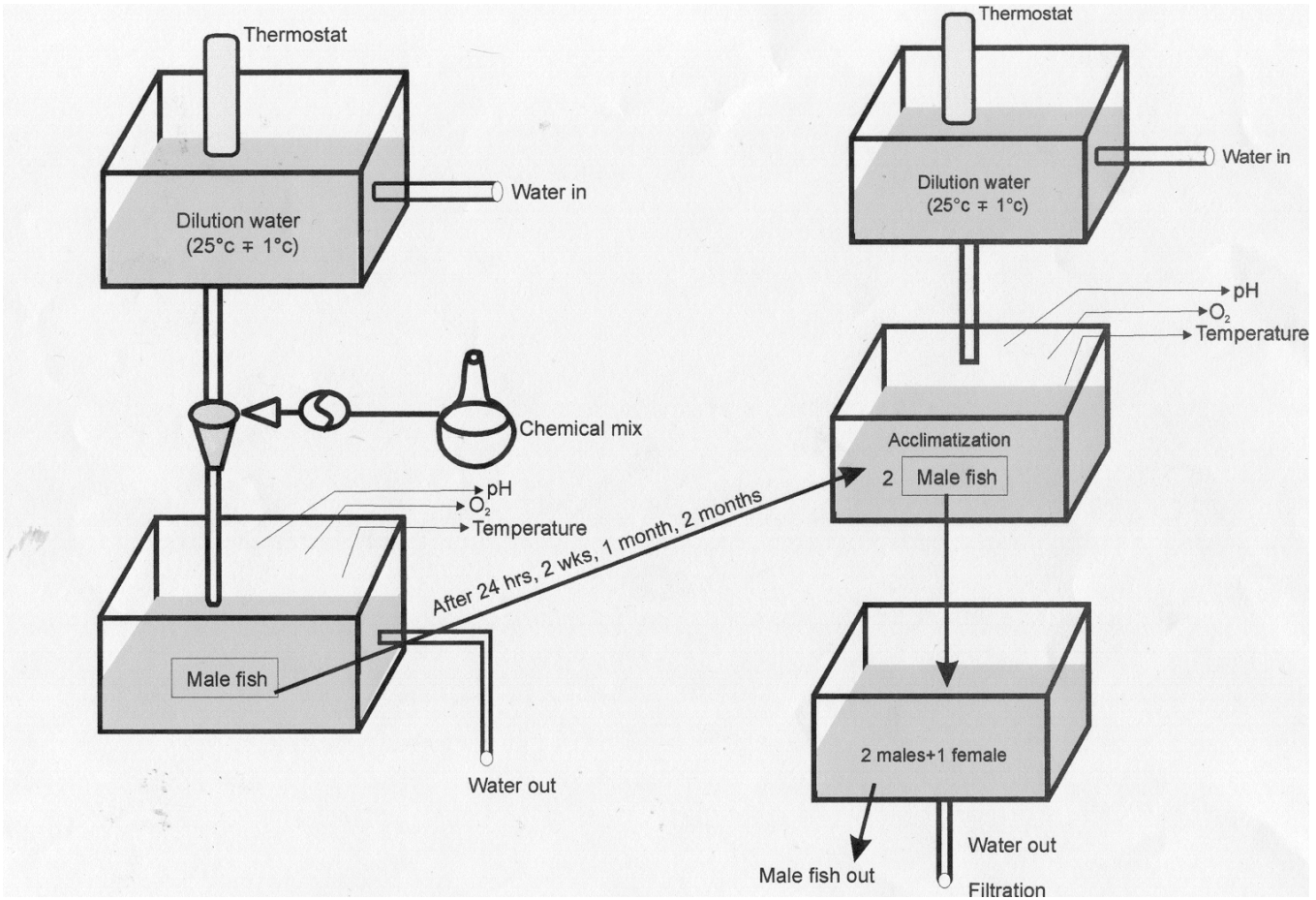
Soon after removal, 5 drops of Rose Bengal were added to the sample for staining the spermatozoa. After 20 minutes, 16 ml of formaldehyde was added to the sample for preservation, after which the sample was passed through a millipore filter (0.22μm pore size) under vacuum. The filter paper containing sperm was dried on a slide warmer, placed on a glass slide, and cleared with immersion oil. The number of sperm was counted under a light microscope at a magnification of 400 × in an area measuring 0.3 × 0.3 mm (0.09 mm^2).

The count was repeated on each 10 randomly selected portions of the filter. The mean value of these readings was used to backcalculate the estimated total number of sperm present in the sample. I ran three tests for each tested concentrations on three different males.

After introducing the a ripe female into an aquarium holding a male conspecific, male activity was monitored, and water samples collected, at different time intervals before egg deposition. The first water sample was removed just before the introduction of the female and served as control, the additional samples were collected every 20min, 40min, 60min, 120min and 180min.

To estimate the duration of sperm activity, males were anesthetized and after gentle abdominal pressure, sperm samples were collected with a pipette directly from the genital papilla. The sperm were then thoroughly mixed with water, a drop of sperm solution was placed under the microscope and sperm movement monitored until most of the sperm stopped moving.

It was also found that “artificial” sperm trails can be easily obtained by gently sqeezing a male’s abdomen while passing his genital papilla over a slide. In this way we could estimate the life span of the sperm by keeping inside a beaker filled with 2 litres of water, maintained at 21°c and observed every 10 minutes under a microscope.



FigureB: Exposure of male zebrafish to the chemicals under flow-through conditions before the mating events.

2.6) Ultrastructure of the testis and spermiogenesis

Zebrafish (*B rerio*) are small-to-medium-sized fishes which measure 3-5 cm in the adult state. In the laboratory, male fishes were killed by gently pressing their head. Under a stereo microscope, testis were dissected and fixed in 5% glutaraldehyde in 0.1M phosphate buffer with ph 7.4. The fixed tissues were rinsed in buffer and postfixed in 1% osmium tetroxide in phosphate buffer. After this fixation, the tissues were prepared for embedding and passed through each stage described on the following shematical representation:

3 x 10min washed in PBS (phosphate buffer + sucrose)

↓

Ethanol series:

30, 50, 70, 90, and 100% acetone each 2x 10 min.

↓

(1) 100% acetone / (2) Pure Araldite

2:1 (v / v)

1:2 (v /v) each 30 min.

↓

Pure Araldite (allow to stay all night long without covering the flasks)

↓

The next day place the samples with pure Araldite in the forms

↓

leave stay for 48 hrs at 50°c in an oven .

The embedding mixture (pure Araldite):

The pure Araldite was obtained by mixing:

50 ml of Araldite M

50 ml of Araldite M Hardener 964, and

2 g of Araldite M Accelerator 960.

Microsections of the samples thus embedded after 48 hrs were performed using an ultramicrotome Leicer UCT and which afterwards were colored with methylene blue. The 0.5µ sections were made for the observation under light microscope and 0.1µ sections for the transmission electron microscope (TEM) Zeiss EM 902.

3) Results

3.1) Exposure of zebrafish to the pesticides and their mixtures

3.1.1) Exposure to DDT

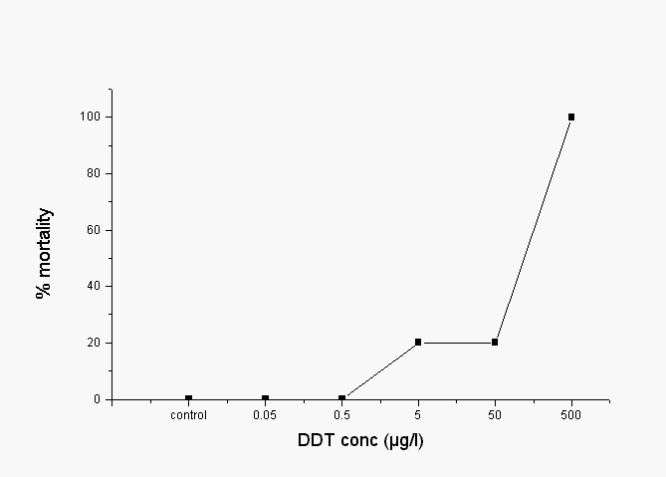


Figure 1a: Rate of mortality of zebrafish within 4 days of exposure to DDT at each of the tested concentrations.

It is difficult to establish the 96-hrs LC50, because at none of the tested concentrations is the half population size of the fish died. But at the highest concentration is already 100% of the fish population died.The half fish population is succumbed already at the concentration of 50µg/l on the 18th day. At 5µg/l the rate of mortality increased to 30% on the 7th day and to 40% from the 14th day.

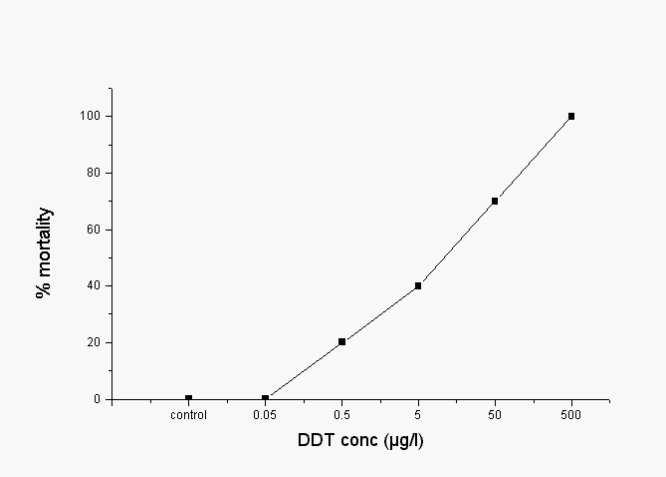


Figure 1b: *Rate of mortality of zebrafish within 21 days of exposure to DDT at each of the tested concentrations*

3.1.2) Exposure to Arochlor 1254

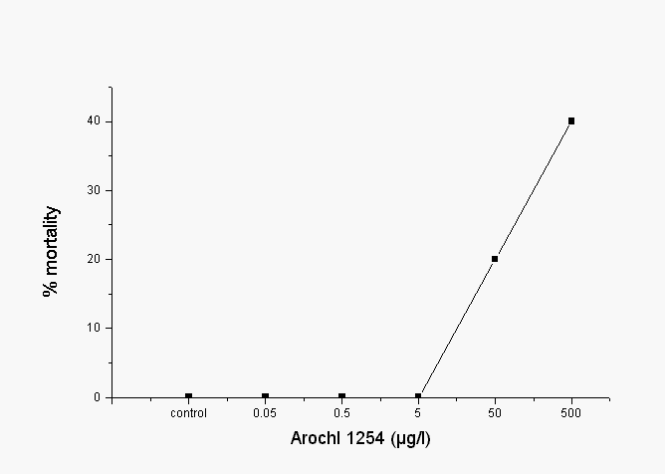


Figure 2a: Rate of mortality of zebrafish within 4 days of exposure to Arochlor 1254 at all the tested concentrations.

The LC50's can not be established, because at none of the tested concentrations is the half population of the test organisms succumbed. And at 500µg/l which is the highest PCB's tested concentration only 40% mortality is recorded.

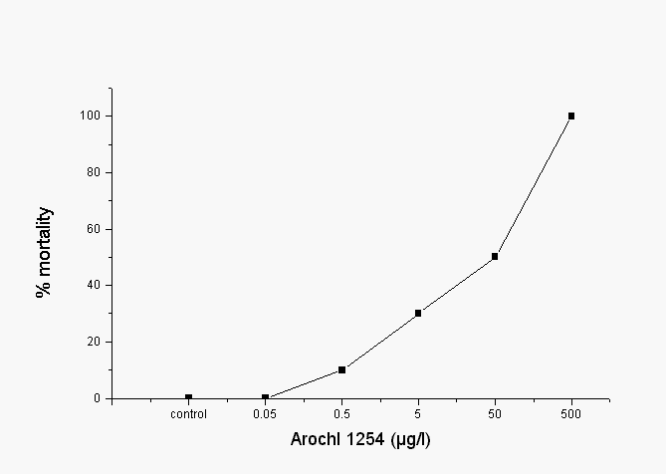


Figure 2b: Rate of mortality of zebrafish within 21 days of exposure to Arochlor 1254 at all the tested concentrations.

The half of the population died on the 17th day at 50µg/l and from the 12th day at 500µg/l, a 100% mortality is recorded. But the effect of Arochlor 1254 on zebrafish seems to be milder and slower than that of DDT. This result can be also emphasized by the rate of mortality recorded at 0.5µg/l only on the 20th day.

3.1.3) Exposure to the 1:1 mixture of DDT and Arochlor 1254

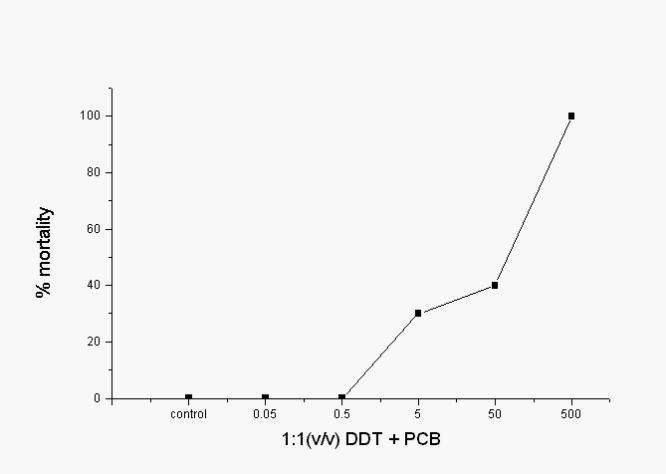


Figure 3a: Rate of mortality of zebrafish exposed within 96 hours to the 1:1 mixture DDT and Arochlor 1254 at each of the tested concentrations.

30% and 40% mortality are already recorded at the concentrations 5 and 50µg/l respectively bigger than when the chemicals are applied alone. And at the highest concentration a 100% mortality is already reached.

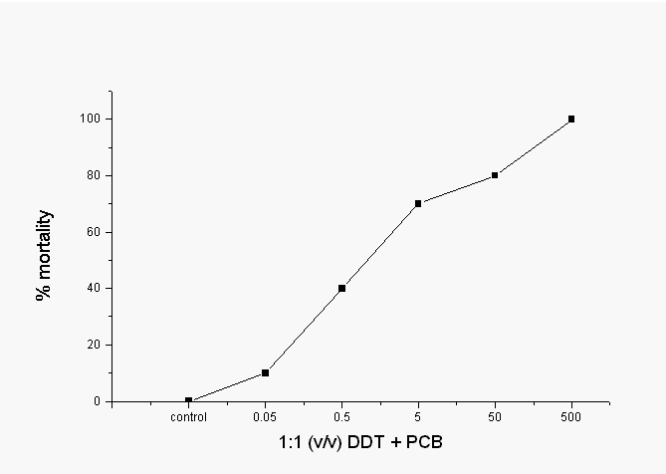


Figure 3b: *Rate of mortality of zebrafish within 21 days of exposure to the mixture DDT and Arochlor 1254 at each of the tested concentrations*.

In contrast to the other results, a 10% mortality has been recorded at the lowest tested concentration 0.05µg/l. And this rate of mortality is greater at the other tested concentrations than that recorded in the other cases. These results can lead us to thing about a synergical activity between DDT and Arochlor 1254.

3.2) Bioaccumulation

3.2.1) Static fish test with DDT

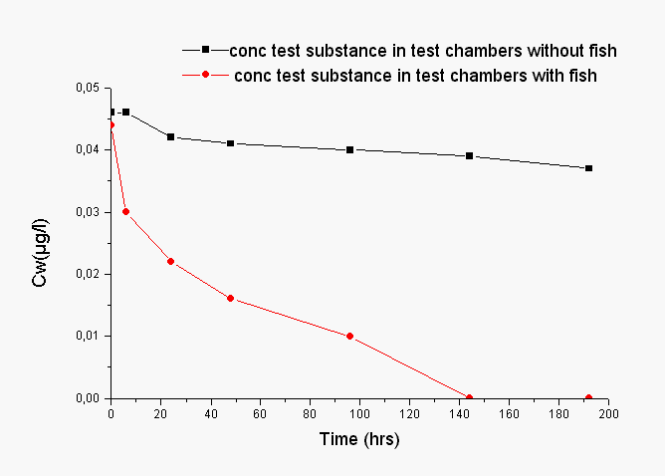


Figure 4a: *Graph DDT concentrations in the medium, Cw versus time within 8 days in aquaria with and without fish at the tested concentration 0.05µg/l.*

No equilibrum is reached. BCF 8days cannot be determined because the test substance is not more detectable after 144 hours of exposure.The minimum BCF = 756.76

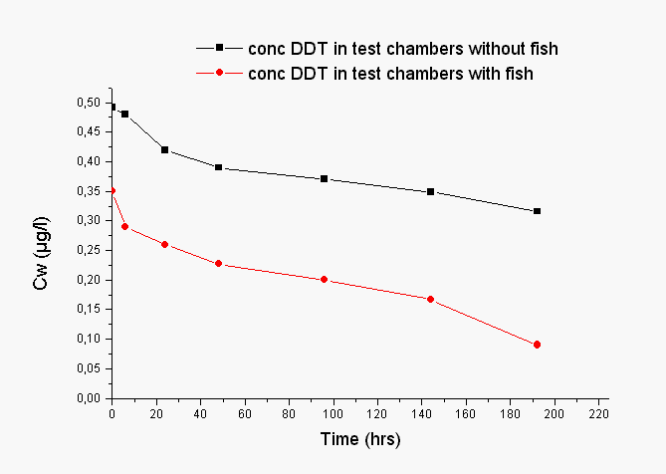


Figure 4b: *Graph of DDT concentration in the medium (Cw) versus time in aquaria with and without fish within 8 days at the tested concentration 0.5µg/l.*

No equilibrum is reached

BCF 8 days = 654.33

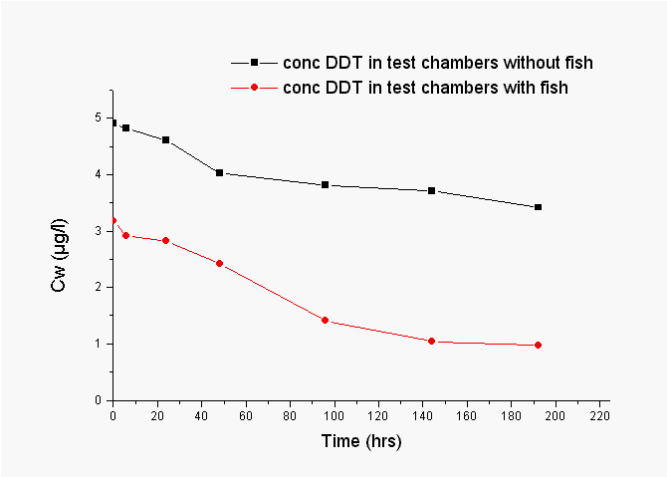


Figure 4c: *Evaluation of DDT residues in the medium with and without fish at 5µg/l dosed concentration*.

No equilibrum is reached

BCF 8days = 752.22

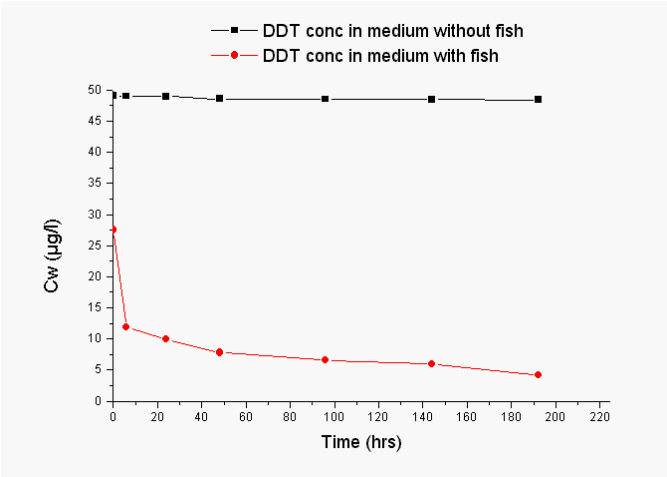


Figure 4d: *Evaluation of DDT residues in medium with and without fish within 8 days at 50µg/l initial dosed concentration.*

No equilibrum is reached

BCF8days =843.42

3.2.2) Static fish test with Arochlor 1254

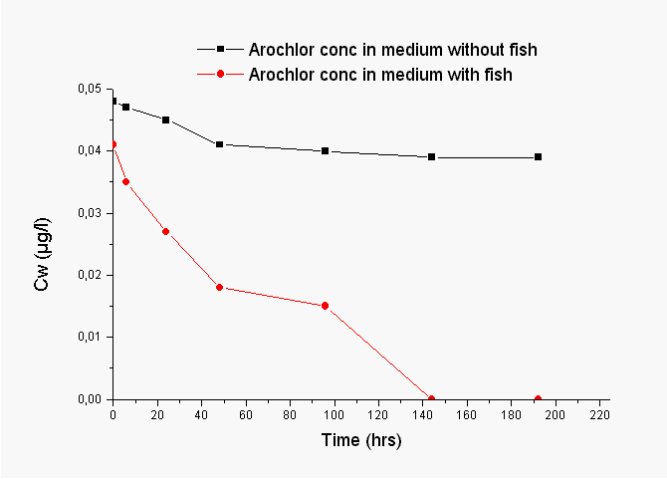


Figure 5a: *Evaluation of Arochlor 1254 residues in the medium and fish at 0.05µg/l initial dosed concentration.*

No equilibrum is reached

No BCF 8days can be determined because the test substance is not more detectable from 144 hours after exposure

The minimum BCF = 784.36

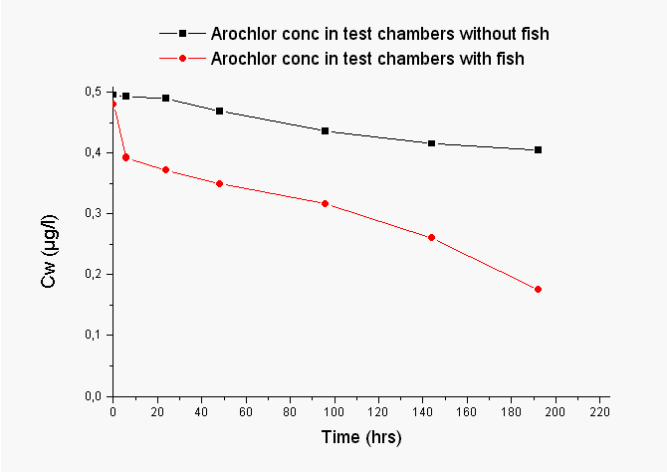


Figure 5b: *Evaluation of Arochlor residues in the medium with and without fish within 8 days of exposure at 0.5µg/l initial dosed concentration.*

-No equilibrum is reached

-BCF 8days = 439.94

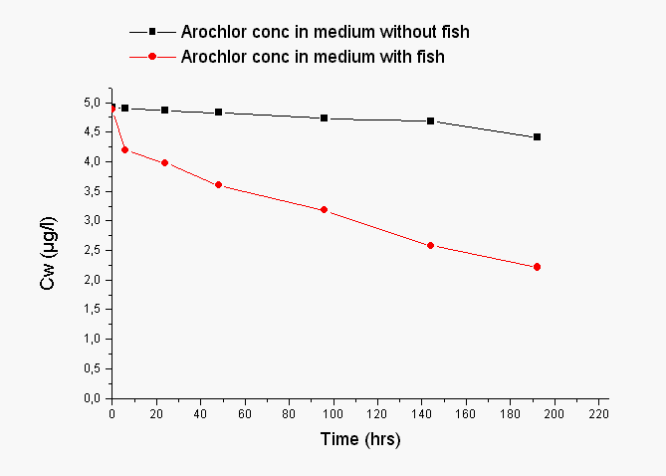


Figure 5c: *Evaluation of Arochlor residues in the medium with and without fish within 8days of exposure at 5µg/l initial dosed concentration*.

No equilibrum is reached

BCF 8days = 722.28

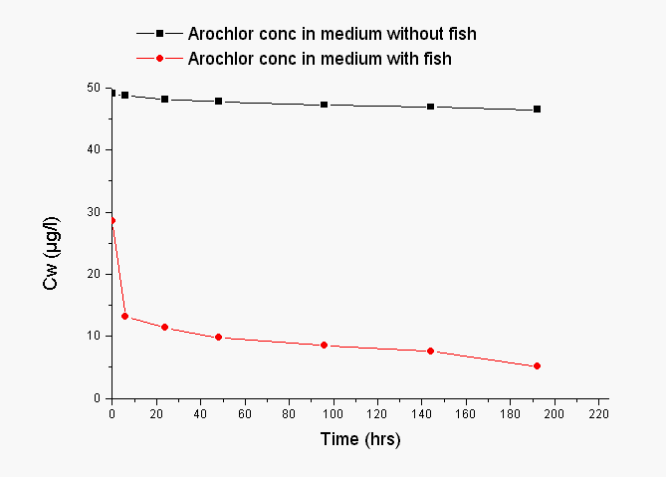


Figure 5d: *Evaluation of Arochlor residues in the test chambers with and without fish within 8 days of exposure at 50µg/l initial dosed concentration*.

No equilibrum is reached

BCF 8days = 785.47

3.2.3) Life cycle stages (LCS) of the juvenile zebrafish emerged.

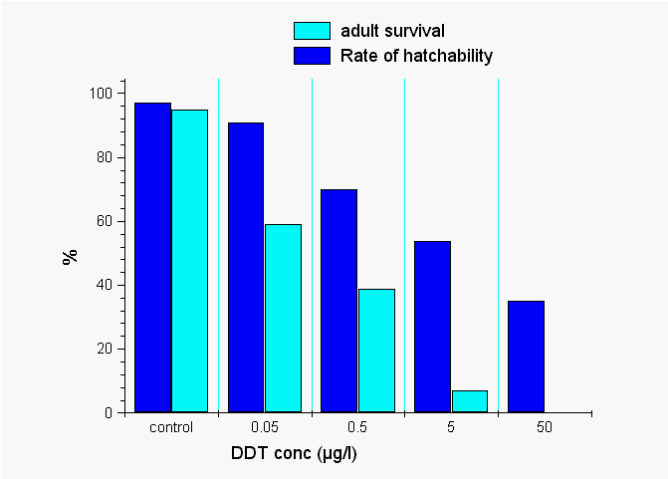


Figure 6: *Rates of hatchability and adult survival in the F-I generation at the different DDT tested concentrations.*

At 50µg/l , all the fish succumbed before the F-II generation.

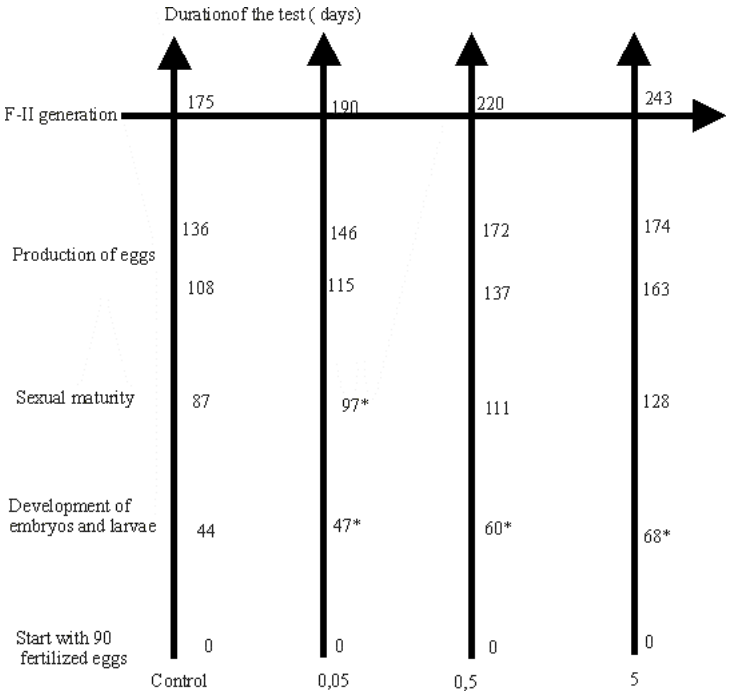


Figure 7: *Duration of the LCS up to 6 weeks in the F-II generation of the juvenile zebrafish at the different DDT concentrations*.

* indicates where more than 10% mortality has been recorded between development of embryos and larvae and the sexual maturity.

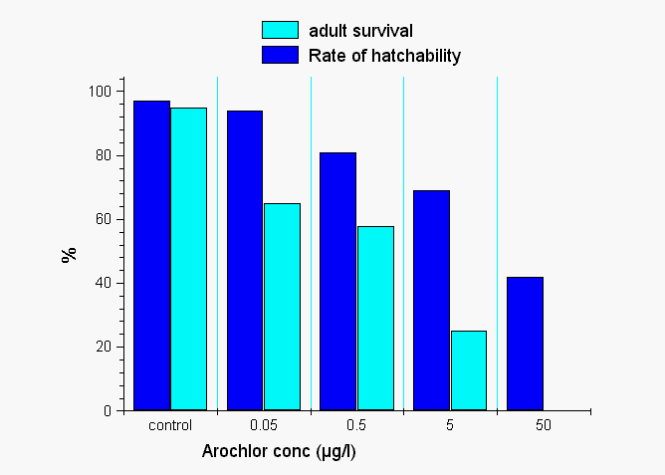


Figure 8: *Rates of hatchability and adult survival in the F-I generation at the Arochlor 1254 tested concentrations*.

All the fish succumbed before the F-II generation at the tested concentration of 50µg/l.

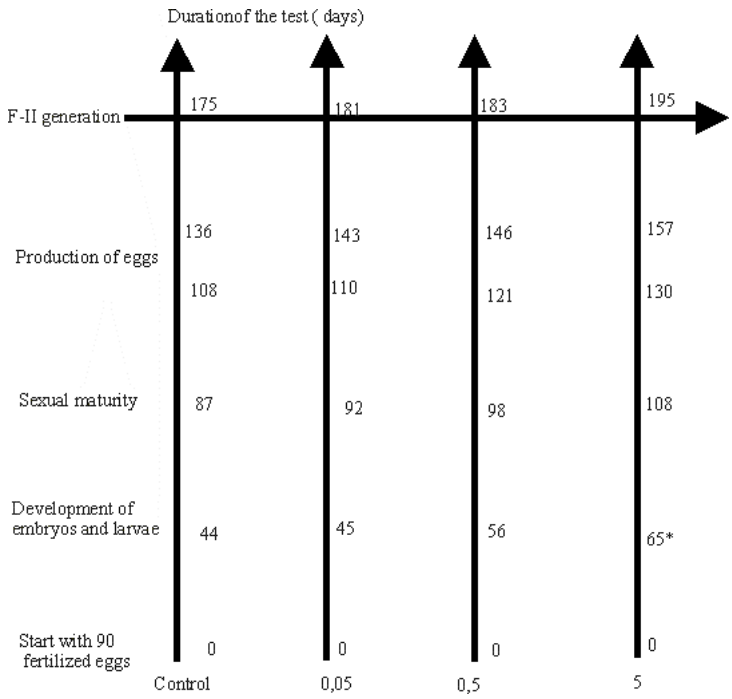


Figure 9: *Duration of the LCS up to 6 weeks in the F-II generation of the juvenile zebrafish emerged at the different Arochlor 1254 tested concentrations*.

* indicates where more than 10% mortality has been recorded between the development of embryos and larvae and the sexual maturity.

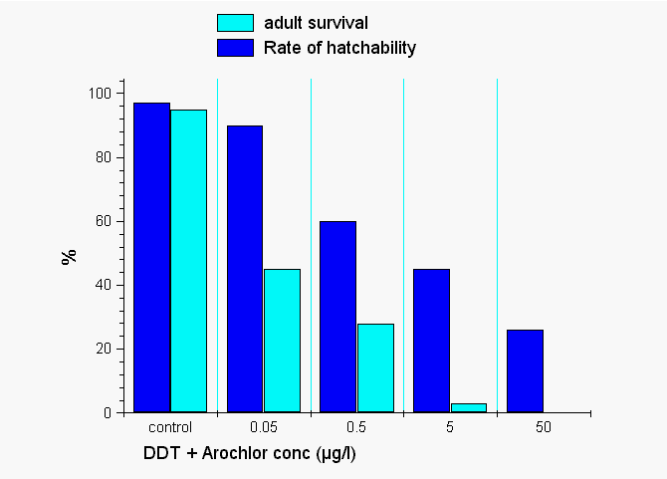


Figure 10: Rates of hatchability and juvenile survival in the F-I generation at the different (1:1) DDT + Arochlor 1254 mixture tested concentrations.

All the fish succumbed before the F-II generation at the tested concentration 50µg/l.

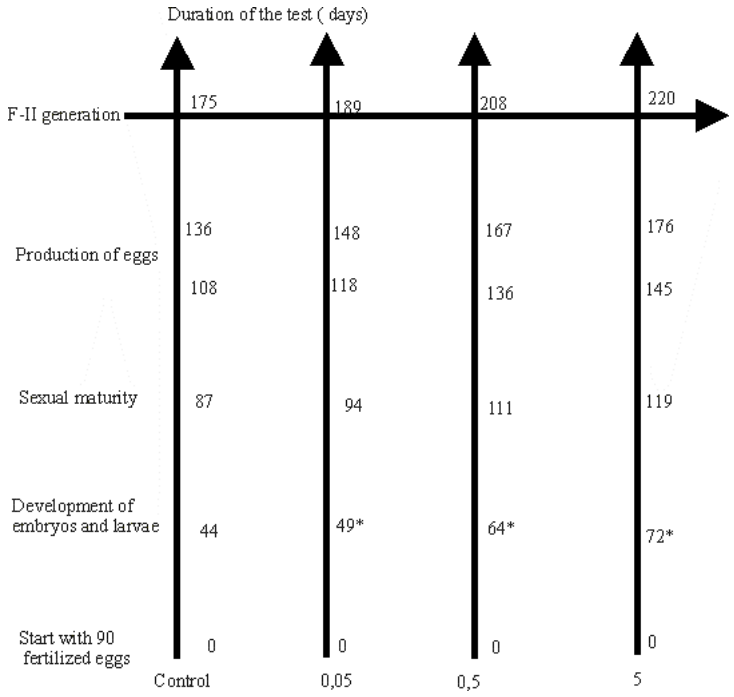


Figure 11: *Duration of the LCS up to 6 weeks in the F-II generation of the juvenile zebrafish emerged at the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations.*

indicates where more than 10% mortality has been recorded between development of embryos and larvae and the sexual maturity.

More than 10% mortality has been recorded at all the tested concentrations.

3.2.4) Evaluation of the length of zebrafish after embryos and larval stage

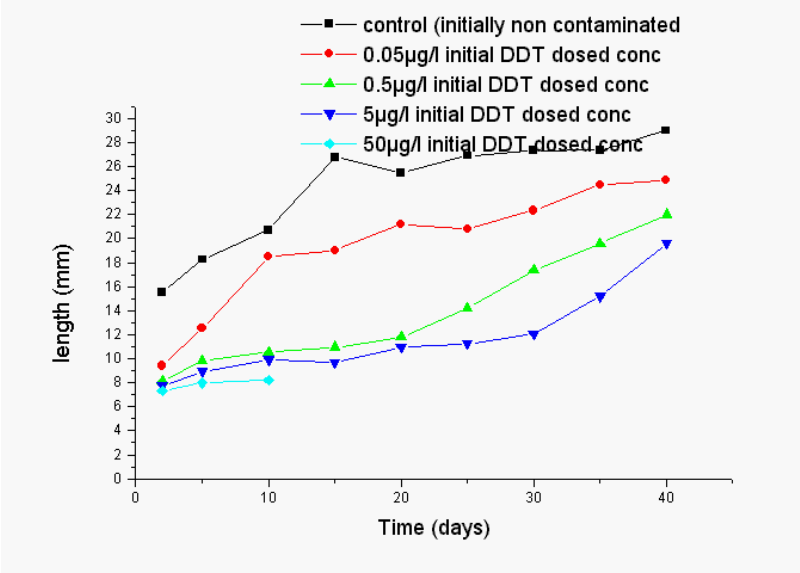


Figure 12a: *Average length of juvenile zebrafish emerged at the different DDT tested concentrations after the development of embryos and larvae within 40 days.*

At the tested concentration 50µg/l, all the fish succumbed after 10 days.

F-ratio = 1.37 at df (4, 40)

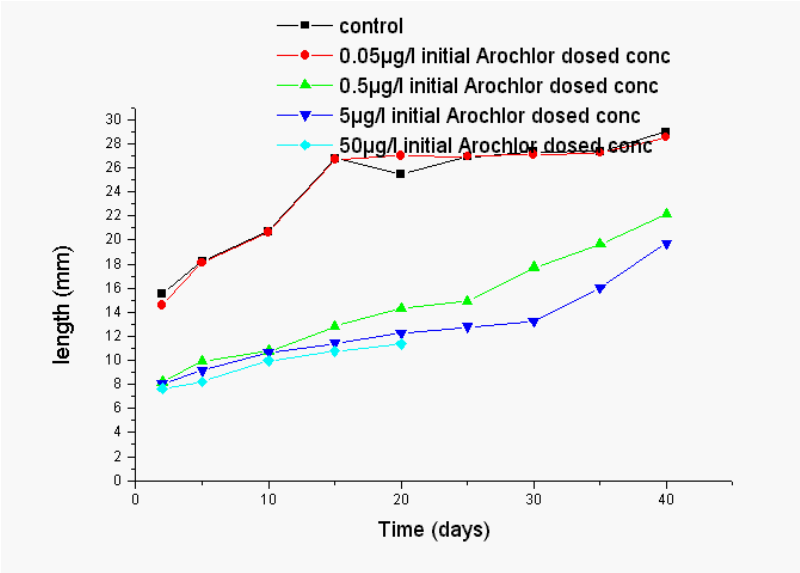


Figure 12b: *Average length of juvenile zebrafish emerged at the different Arochlor 1254 tested concentrations within 40 days after the development of embryos and larvae.*

But in this case, at the tested concentration 50µg/l, all the fish succumbed only after 20 days.

F-ratio = 1.65 at df (4, 40)

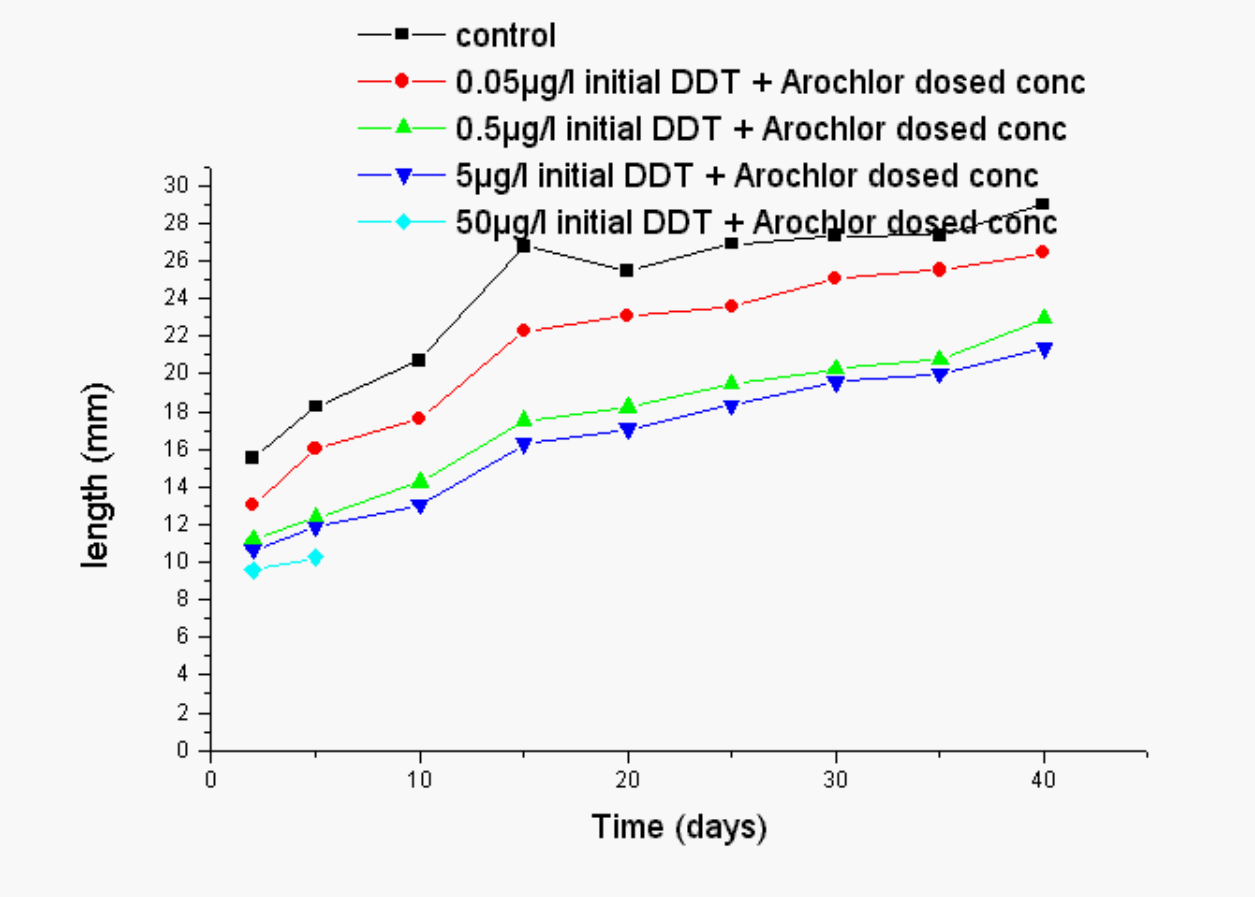


Figure 12c: *Average length of zebrafish emerged at the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations within 40 days after the development of embryos and larvae.*

All the fish have already succumbed after 5 days.

F-ratio = 0.88 at df (4, 40)

3.3) Sperm count after mating events of male zebrafish

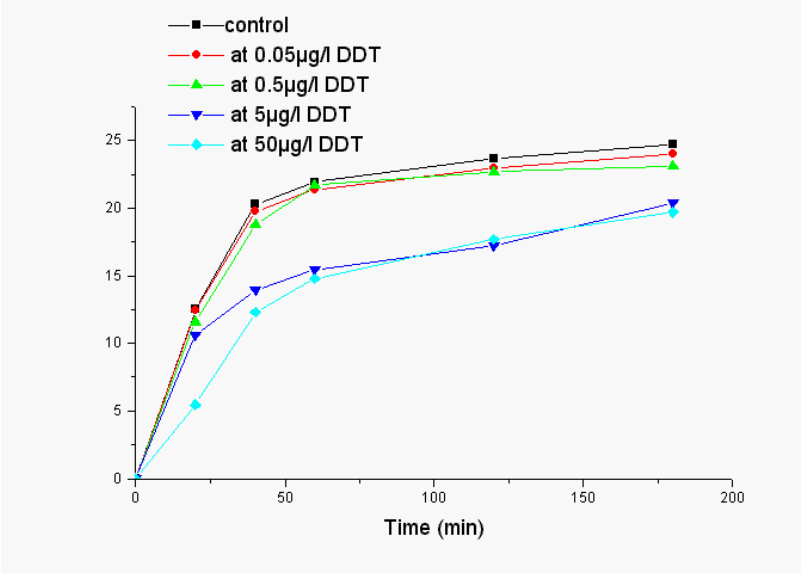


Figure 13a: Sperm count (10 ^{3} sperm /ml) during mating events of male zebrafish after 24 hours of exposure to different DDT tested concentrations. F-ratio = 0.11 at df (4, 20).

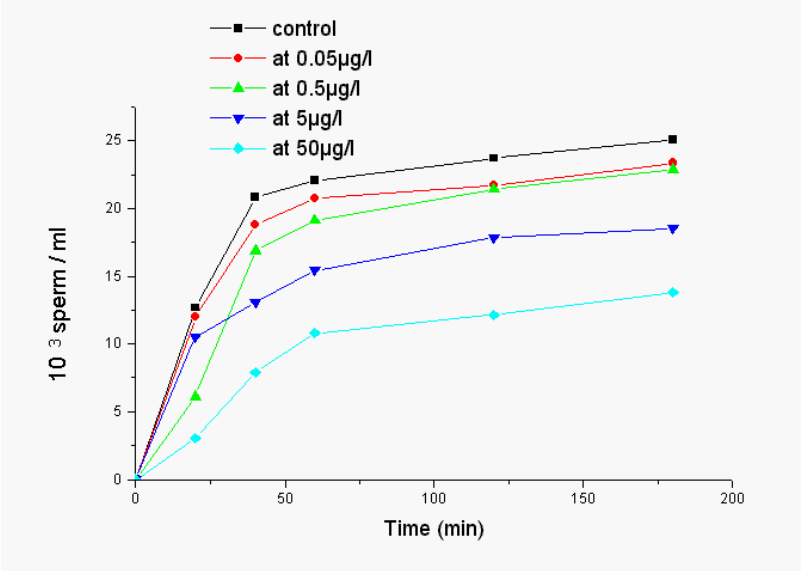


Figure 13b: sperm counts (10 ^{3} sperm / ml) during mating events of zebrafish after 2 weeks of exposure to DDT at the different tested concentrations. F-ratio = 1.35 at df (4, 20).

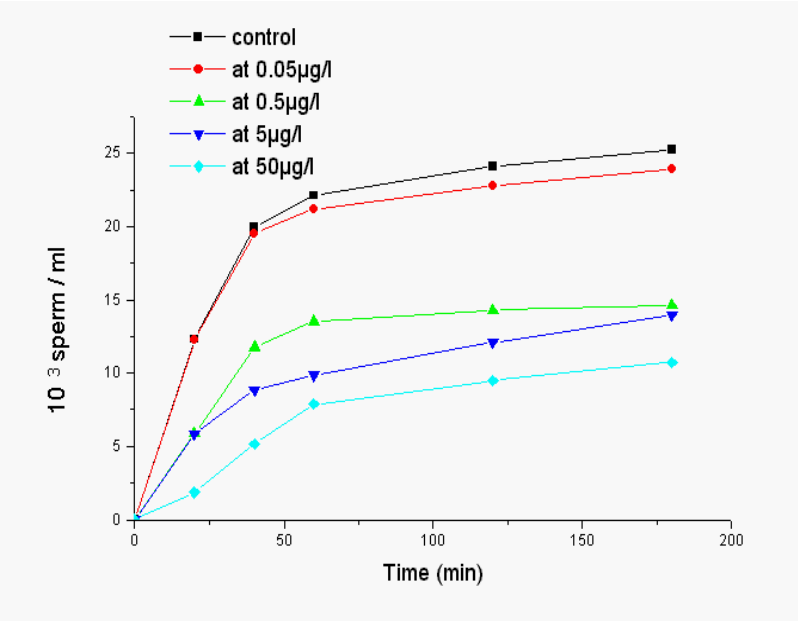


Figure 13c: Sperm count (10 ^{3} sperm / ml) during mating events of male zebrafish after 1 month of exposure to DDT at the different tested concentrations.F-ratio = 0.71 at df (4, 20).

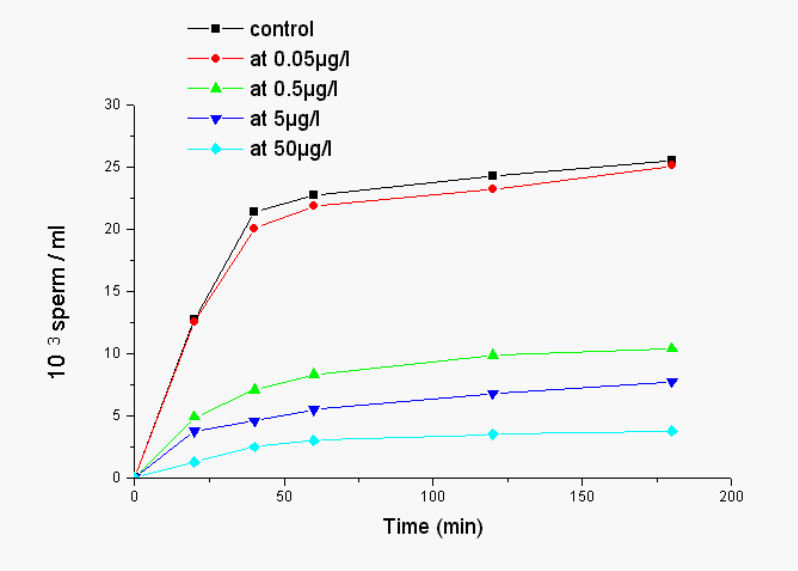


Figure 13d: Sperm count (10 ^{3} sperm / ml) during mating events of male zebrafish after 2 months of exposure to the different DDT tested concentrations. F-ratio = 0.31 at df (4, 20).

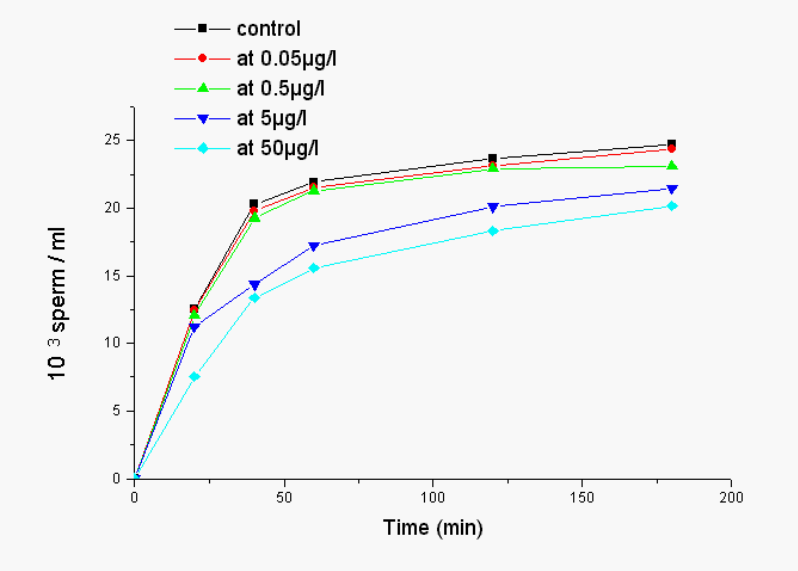


Figure 14a: *Sperm count (10^3 / ml) during mating events of zebrafish after 24 hours of exposure to the different Arochlor 1254 tested concentrations.*
F-ratio = 0.07 at df (4, 20).

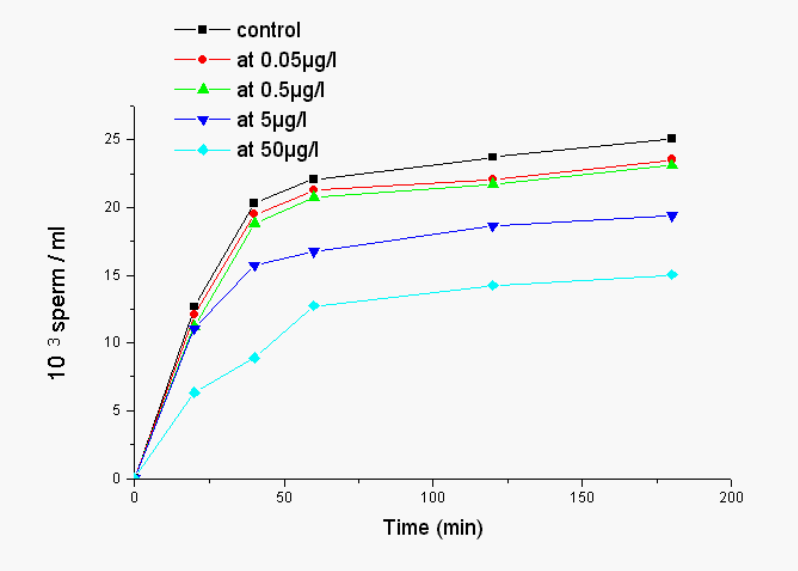


Figure 14b: *Sperm count (10^3 sperm / ml) during mating events of zebrafish after 2 weeks of exposure to different Arochlor 1254 tested concentrations.*
F-ratio = 0.17 at df (4, 20)

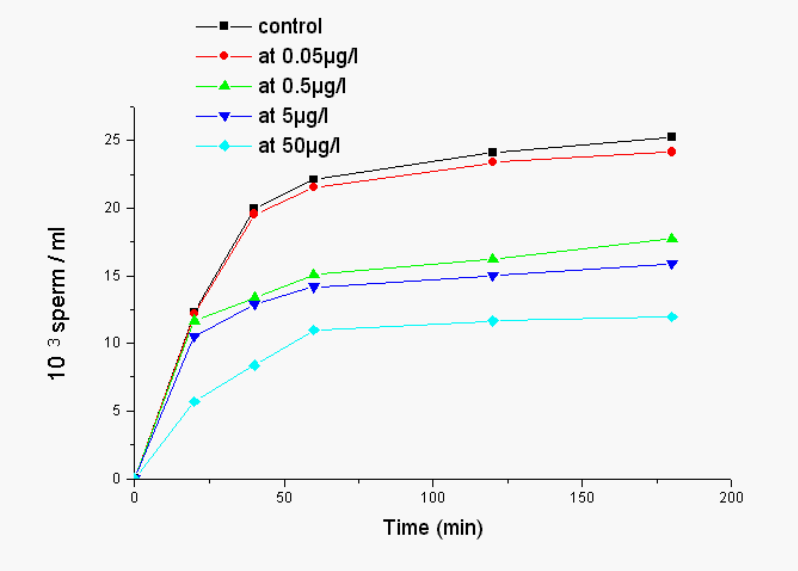


Figure 14c: Sperm count (10^3 sperm / ml) during mating events of zebrafish after 1 month of exposure to the different Arochlor 1254 tested concentrations. F-ratio = 0.33 at df (4, 20).

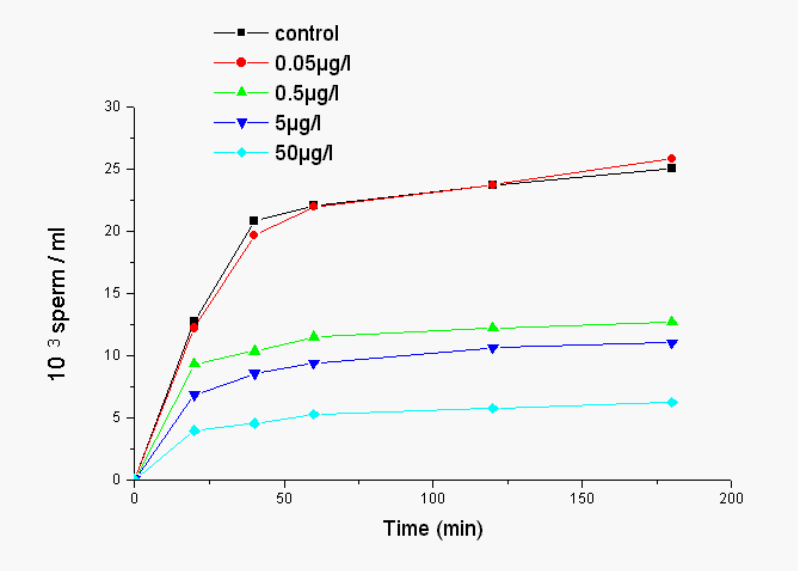


Figure 14d: *Sperm count (10^3 sperm / ml) during mating events of zebrafish after 2 months of exposure to the different Arochlor 1254 tested concentrations.*
F-ratio = 1.04 at df (4, 20).

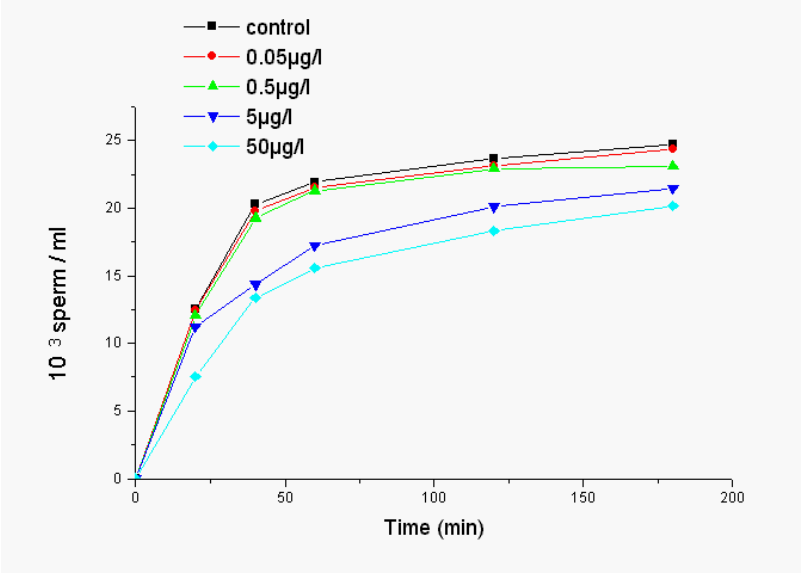


Figure 15a: *Sperm count (10^3 sperm / ml) during mating events of zebrafish after 24 hours of exposure to the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations.*
F-ratio = 0.98 at df (4, 20).

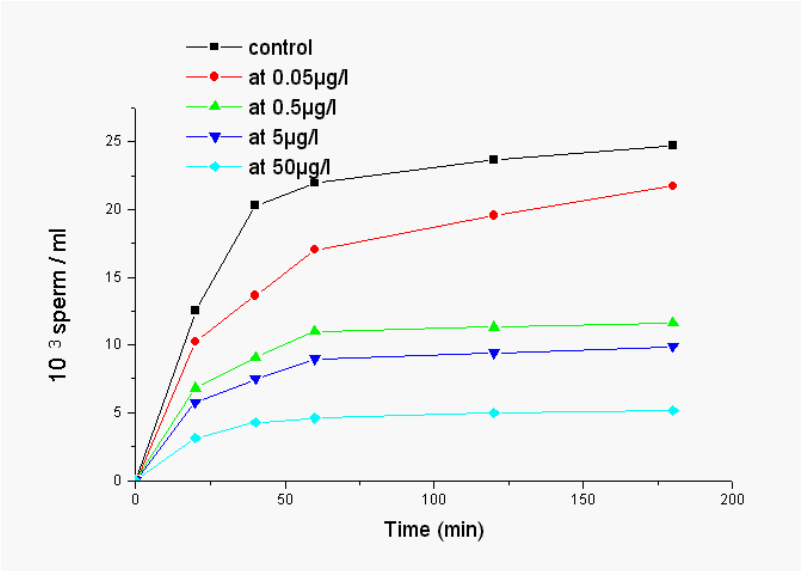


Figure 15b: *Sperm count (10^3 sperm / ml) during mating events of zebrafish after 2 weeks of exposure to the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations.*
F-ratio = 1.05 at df (4, 20).

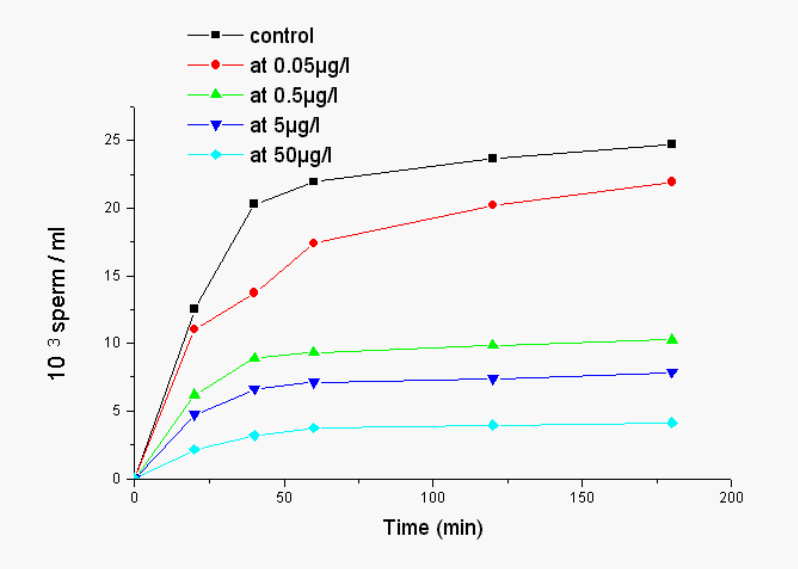


Figure 15c: Sperm count (10^3 sperm / ml) during mating events of zebrafish after 1 month of exposure to the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations. F-ratio = 1.58 at df (4, 20).

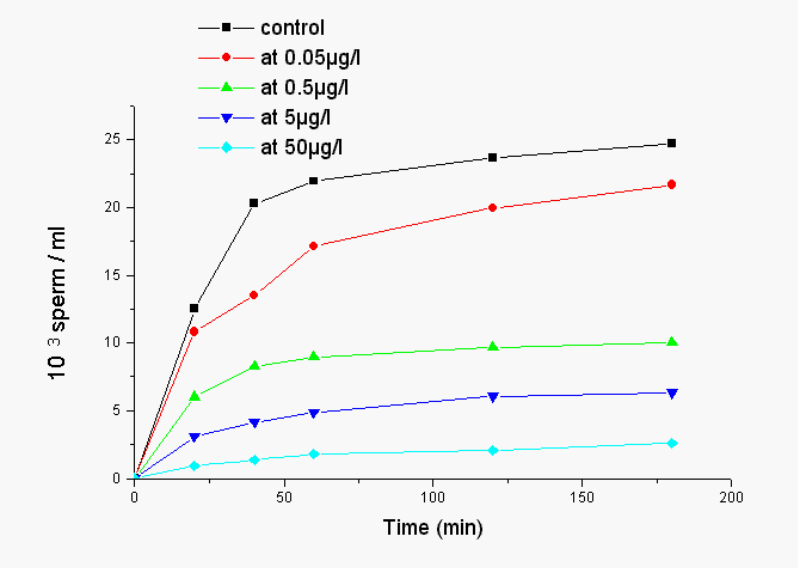


Figure 15d: Sperm count (10^3 sperm / ml) during mating events of zebrafish after 2 months of exposure to the different 1:1 mixture of DDT and Arochlor 1254 tested concentrations. F-ratio = 2.17 at df (4, 20).

3.4) Average life span and activity of sperm released during mating events of zebrafish exposed to the different tested concentrations of DDT, Arochlor 1254 and their 1:1 mixture

	24h	2 wks	1 mth	2 mths
control	138 ± 15	140 ± 13	142 ± 15	139 ± 16
0.05µg/l	138 ± 14	136 ± 14	130 ± 9	135 ± 10
0.5µg/l	135 ±15	120 ± 12	115 ± 7	105 ± 8
5µg/l	128 ±13	106 ± 8	90 ± 6	86 ± 5
50µg/l	125 ± 11	90 ± 9	81 ± 7	70 ± 4

Table 1a: *Average life span of sperm (in minutes) released during mating events after 24 hours, 2 weeks, 1 month and 2 months of exposure to all the tested DDT concentrations.*
(X ± SE, N = 7)

	24h	2wks	1mth	2mths
Control	28.7 (4min30s)	29.8 (4min15s)	29.7 (4min55s)	30.3 (4min30s)
0.05 µg/l	28.4 (4min40s)	28.7 (4min)	29.2 (4min10s)	29.5 (4min15s)
0.5 µg/l	28.2 (4min35s)	27.6 (4min5s)	28.1 (4min)	26.2 (4min)
5 µg/l	26.3 (4min7s)	25.1 (4min22s)	23.8 (4min19s)	21.9 (4min12s)
50 µg/l	26.1 (4min18s)	22.2 (4min16s)	21.7 (4min20s)	20.1 (4min25s)

Table 1b: *Sperm activity (in minutes) released during mating events of zebrafish after 24 hours, 2 weeks, 1 month and 2 months of exposure to all the DDT tested concentrations.*
(X, SD), N = 10.

	24h	2wks	1mth	2mths
0.05µg/l	139 ± 18	141 ± 16	144 ± 17	143 ± 15
0.5µg/l	138 ± 16	124 ± 11	121 ± 10	115 ± 7
5µg/l	136 ± 14	116 ± 12	102 ±8	97 ± 6
50µg/l	129 ± 11	94 ± 10	90 ±7	81 ± 4

Table 2a: *Average life span of sperm (in minutes) released during mating events of zebrafish after 24 hours, 2 weeks, 1month, and 2 months of exposure to all the Arochlor 1254 tested concentrations. (X ± SE, N = 7).*

	24h	2wks	1mth	2mths
0.05µg/l	28.7 (4min30s)	29.1 (4min36s)	29.5 (4min33s)	29.9 (4min21s)
0.5µg/l	28.4 (4min40s)	28.1 (4min30s)	27.4 (4min28s)	27.0 (4min25s)
5µg/l	27.5 (4min36s)	27.2 (4min26s)	26.2 (4min19s)	24.3 (4min15s)
50µg/l	26.7 (4min25s)	22.6 (4min13s)	21.9 (4min8s)	20.4 (4min)

Table 2b: *Sperm activity (in minutes) released during mating events of zebrafish after 24 hours, 2 weeks, 1 month, and 2 months of exposure to all the Arochlor 1254 tested concentrations. (X, SD), N = 10.*

	24h	2wks	1mth	2mths
0.05µg/l	136 ± 14	132 ± 12	127 ± 9	128 ±7
0.5µg/l	130 ± 12	118 ± 10	110 ± 7	103 ± 5
5µg/l	127 ± 10	101 ± 6	87 ±5	82 ± 2
50µg/l	123 ± 11	88 ± 7	76 ± 4	67 ± 2

Table 3a: *Average life span of sperm (in minutes) released during mating events of zebrafish after 24 hours, 2 weeks, 1 month and 2 months of exposure to all the 1:1 mixture of DDT and Arochlor1254 tested concentrations. (X ± SE, N = 7).*

	24h	2wks	1mth	2mths
0.05µg/l	28.2 (4min39s)	28.1 (4min35s)	28.3 (4min22s)	28.5 (4min20s)
0.5µg/l	27.9 (4min18s)	27.5 (3min45s)	27.6 (3min27s)	27.1 (3min20s)
5µg/l	26.2 (4min10s)	24.9 (3min33s)	23.4 (3min28s)	21.2 (3min18s)
50µg/l	25.7 (4min12s)	21.7 (3min17s)	21.1 (3min14s)	19.8 (3min7s)

Table 3b: *Sperm activity (in minutes) released during mating events of zebrafish after 24 hours, 2 weeks, 1 month, and 2 months of exposure to all the 1:1 mixture of DDT and Arochlor 1254 tested concentrations. X (SD), N = 10*

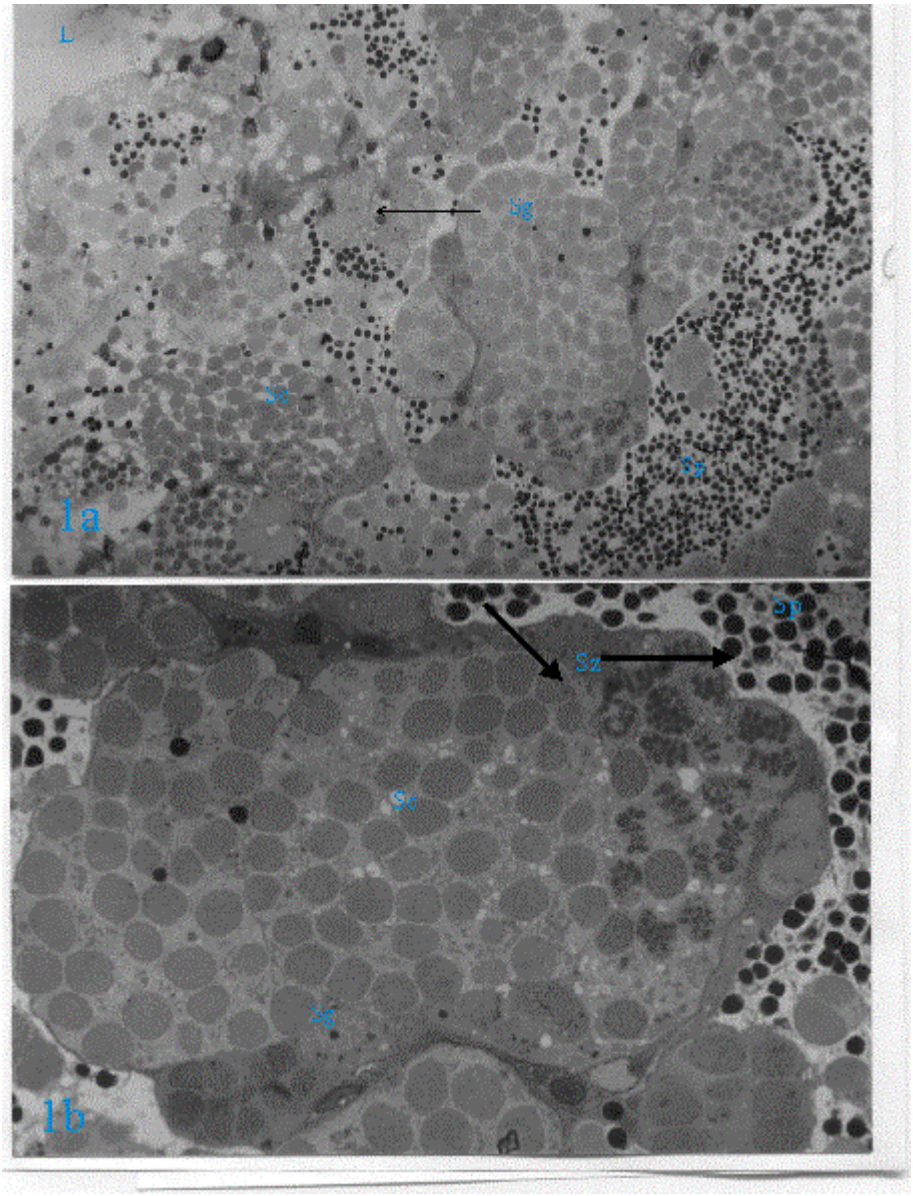
3.5) Organization of the testis and spermiogenesis of male zebrafish exposed to the tested Schemicals

Male zebrafish , *Brachydanio rerio*, have paired testes and no additional reproductive glands. The testes are paired, fusiform structures lying along the lateral body wall on each side from the gill region to the region of the anal fin. A series of about 5 parallel efferent ducts run the length of each testis on its dorsal aspect, collecting matured sperm from the seminiferous tubules. The ducts from the 2 sides course caudally and ventrally to the midline, uniting at the genital papilla. The seminiferous tubules, 50-100μ in diameter form an anastomosing network.

The tubule wall is a simple squamous epithelium bounded by a basement membrane. Lying along the tubule walls and bulging into the sperm filled lumina, are the seminiferous cysts. Each cyst contains the synchronously developing clonal descendants of an original undifferentiated gonial cell. Cyst walls, apparently made of attenuated cystoplasm of Sertoli cells, break down when the cyst is mature, releasing sperm to the tubule lumen.

The following plates show the semi-thin sections of the testes of zebrafish exposed to the different tested concentrations of DDT, Arochlor1254, and their 1:1 mixture within 2 months.

Light microscopy plates



Plates 1a –1b: Semi-thin sections of the non infected zebrafish testes at magnification ×40 and ×100 respectively showing testicular lobule of the germinal epithelium and cylindrical epithelium of the efferent duct. In the lumen, spermatids (Sp) at different developmental stages, numerous spermatocytes (Sc), Spermatogone (Sg) and spermatozoa are present. Spermatogonia possess distinct nucleoli about the same diameter as spermatids in the lobule lumen. Lumen of lobule (L) is often occupied by maturing germ cells.

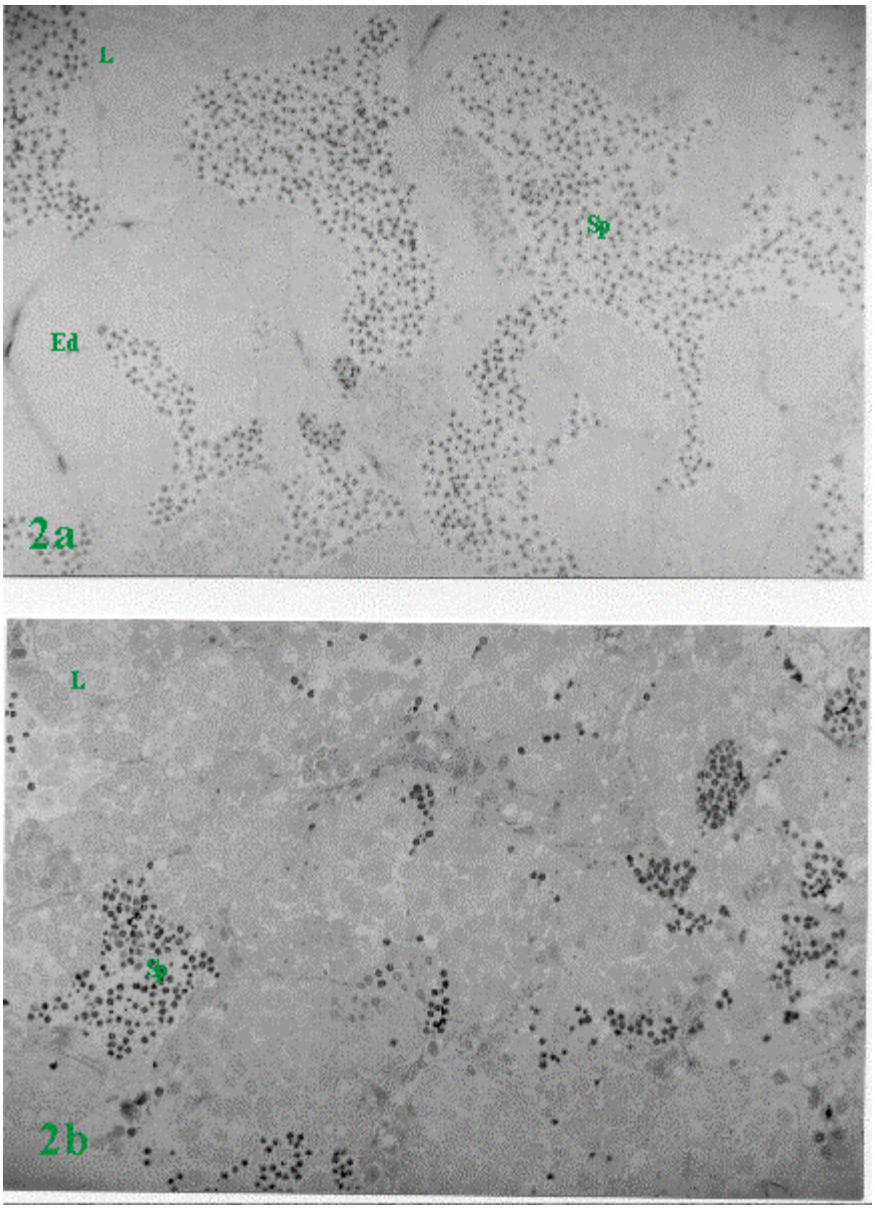


Plate 2a: *Semi-thin sections of zebrafish testes after 1 month of exposure to 0.05µg/l DDT (×10) showing testicular lobule of the germinal epithelium and epithelium of the efferent duct. The lobule lumen (L) is filled with Sp of different developmental stages but showing no great difference with that of the uncontaminated ones.*

Plate 2b: *After 1 month of exposure to 0.5µg/l DDT, the semithin sections of the zebrafish testes showed already a slight reduction in the quantity of Sp present in the lobule lumen although all the other stages observed in that of the non contaminated ones are still present.*

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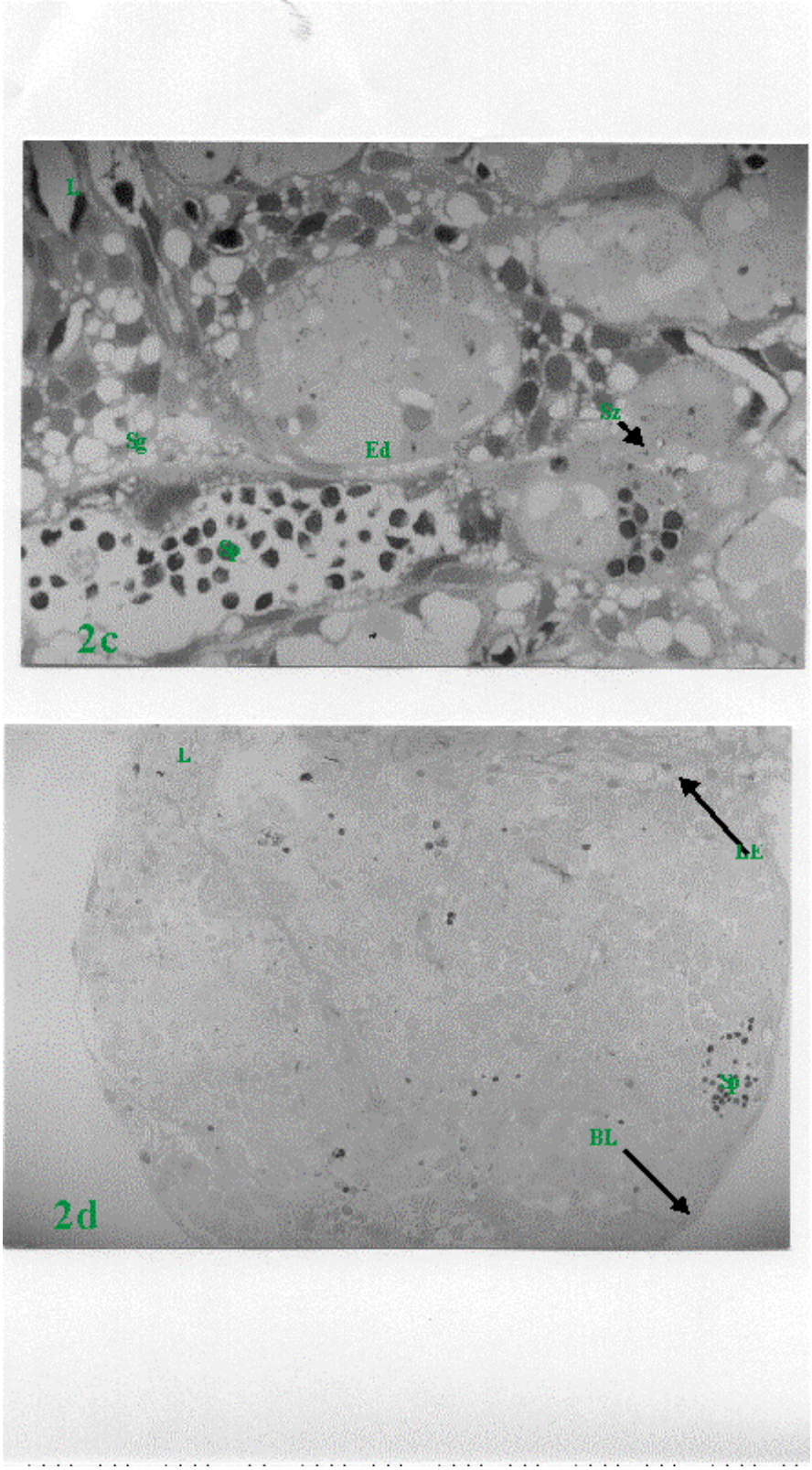


Plate 2c: *After 1 month of exposure to 5µg/l DDT, the semi-thin sections of zebrafish testes still showed a reduction of the Sp content in the lobule lumen. All the other stages of spermatogenesis are present.*

Plate 2d: After 1 month of exposure to 50µg/l DDT, the semi-thin sections of the zebrafish testes showed a great reduction of the Sp content in the lobule lumen. These Sp do not reach the basal lamina (BL) which clearly defines intralobular and interstitial testicular compartmentsas well as Leydig cells (LE).

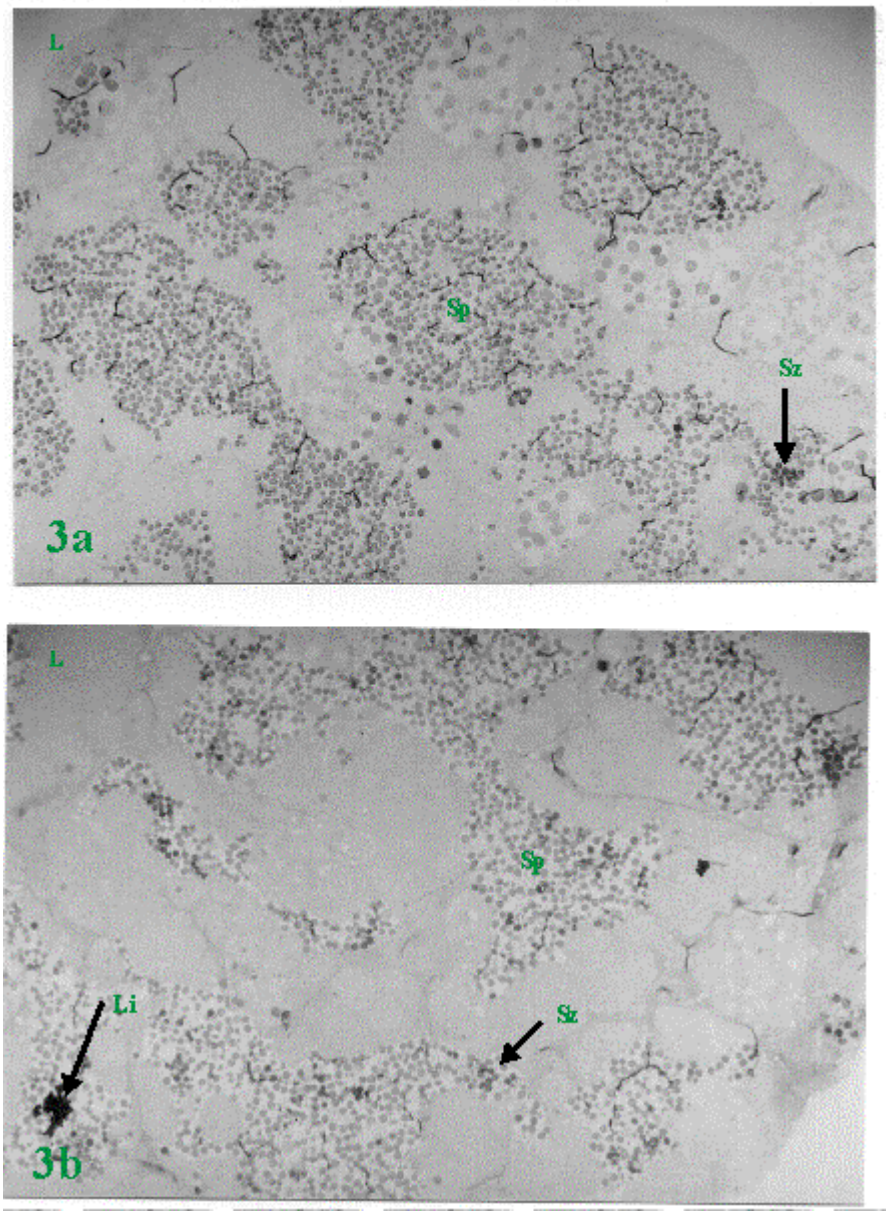


Plate 3a: *After 2 months of exposure to 0.05µg/l DDT, the semi-thin sections of the zebrafish testes show a multitude number of Sp at different developmental stages revealing almost the situation as in those of the non contaminated ones.*

Plate 3b: *After 2 months of exposure to 0.5 µg/l DDT, the semi-thin sections of the zebrafish testes showed also the presence of numerous Sp in the lobule lumen. We could notice the presence of of some lipid droplets.*

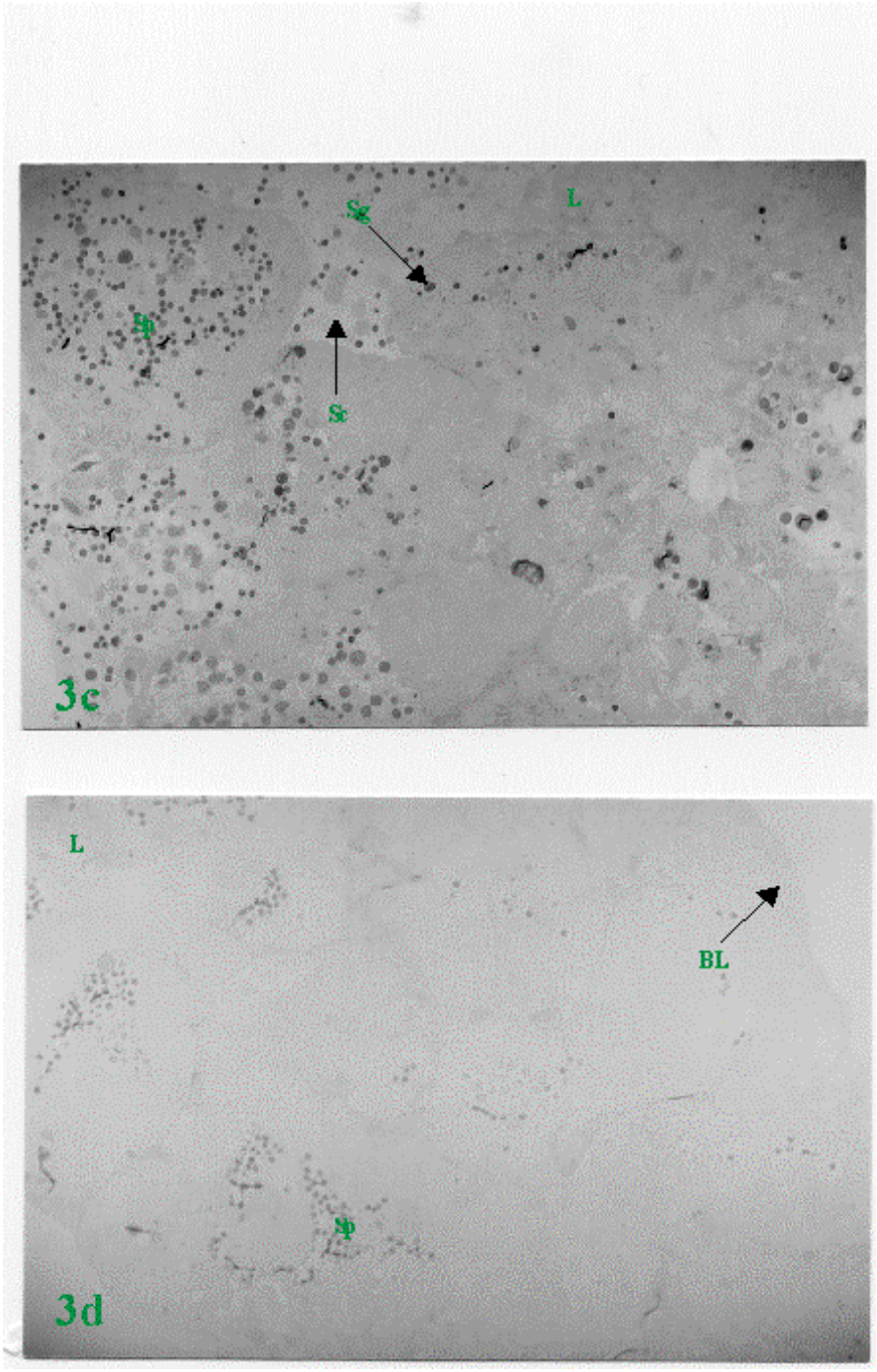


Plate 3c: After 2 months of exposure to 5µg/l DDT, semi-thin sections of zebrafish testes showed also a slight reduction in the number of Sp present in the lobule lumen. Sg and Sc are also present.

Plate 3d: After 2 months of exposure to 50µg/l DDT, semi-thin sections of zebrafish testes revealed a significant decrease of Sp in the lumen and a condensation of the efferent duct lobules with well delimited membranes.

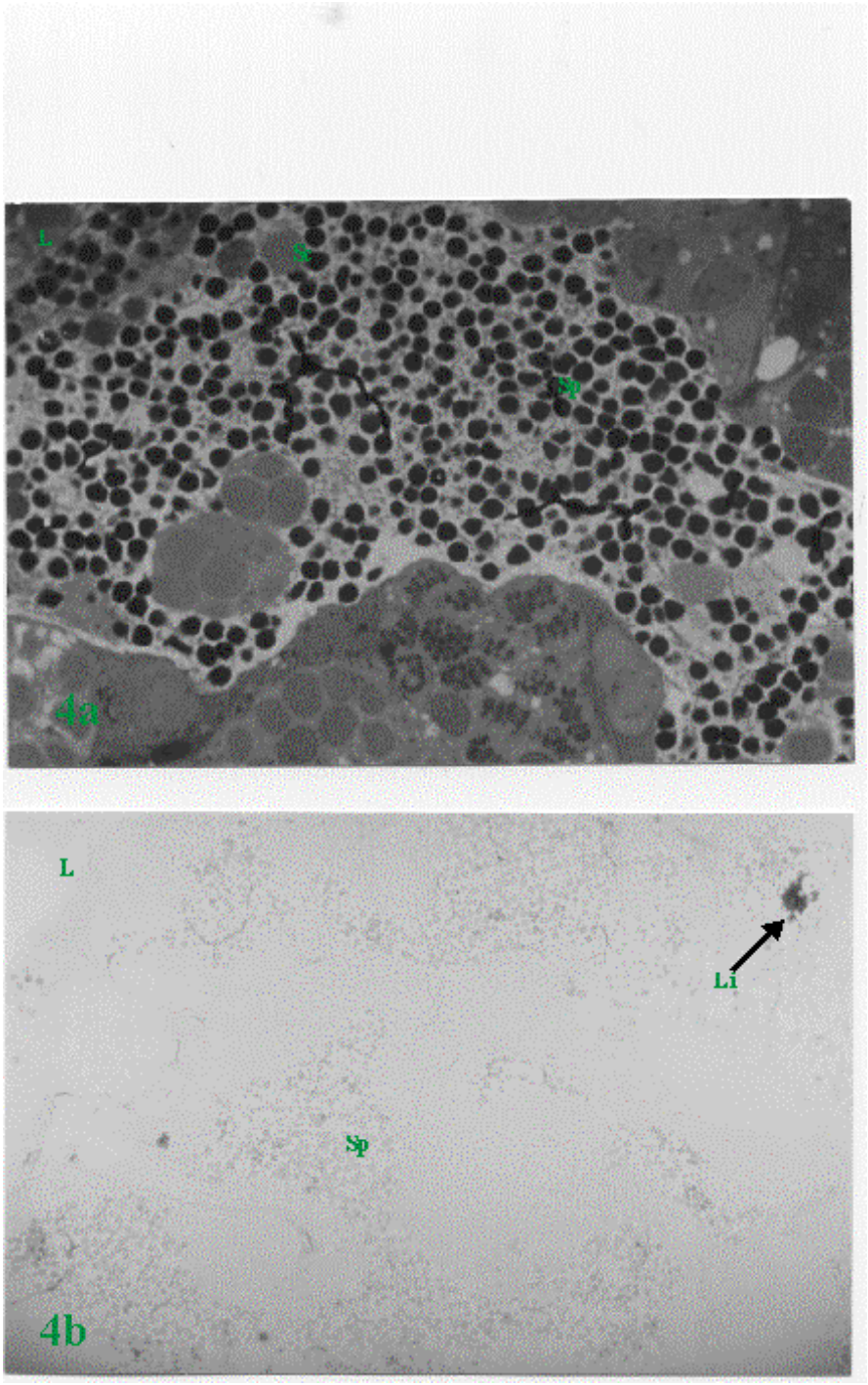
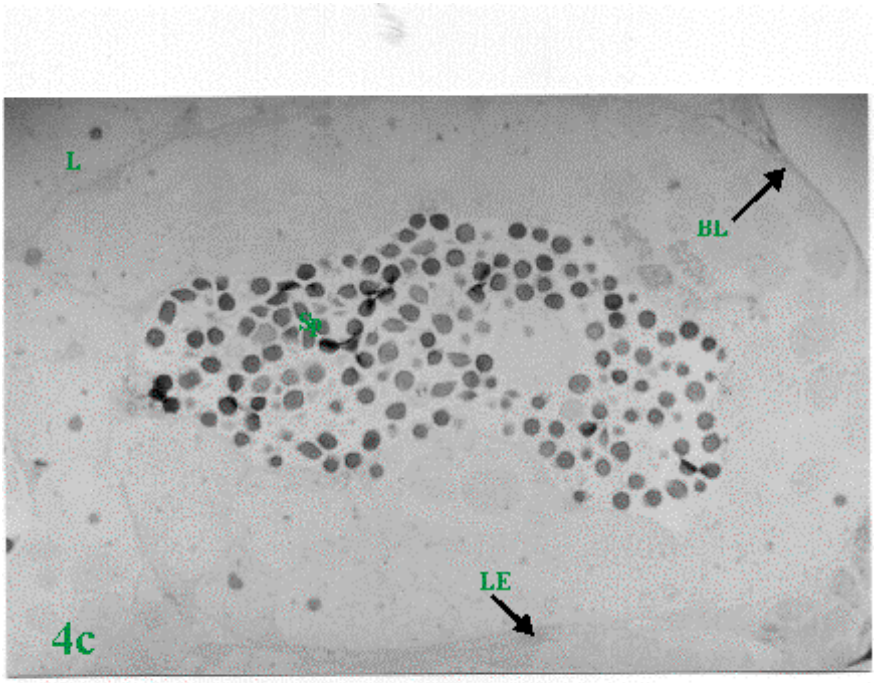


Plate 4a: *Semi-thin sections of the zebrafish testes exposed to 0.05 µg/l Arochlor 1254 after 1month. The lobule lumen is filled with Sp at different developmental stages.*

Plate 4b: *After 1 month of exposure to 0.5µg/l Arochlor 1254, the semi-thin sections of the zebrafish testes showed the lobule lumen filled side by side with Sp at different developmental stages. A slight reduction of their number could be mentioned.*



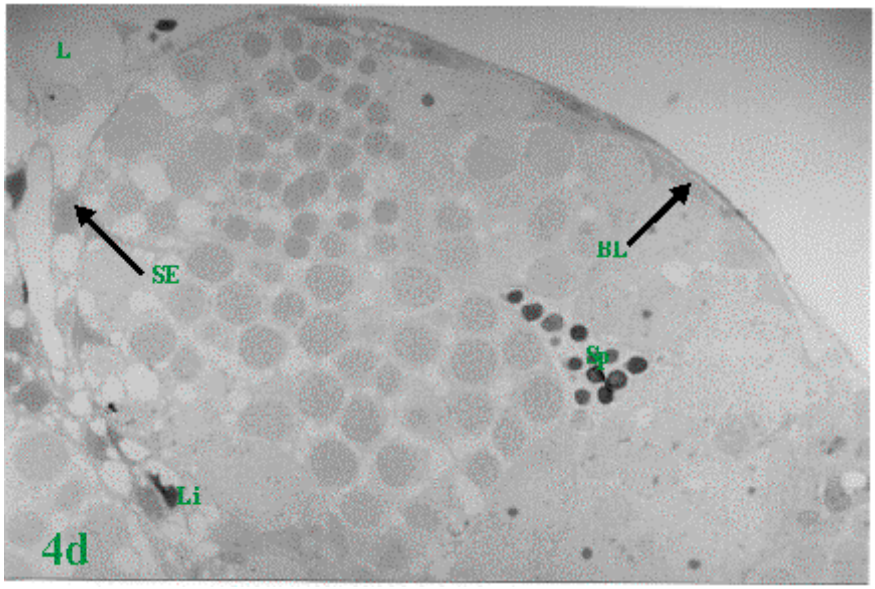


Plate 4c: *After 1 month of exposure to 5µg/l Arochlor 1254 semi-thin sections of the zebrafish testes showed a reduction in the number of Sp at different developmental stages in the lobule lumen although all the other stages of spermatogenesis were still present.*

Plate 4d: *After 1 month of exposure to 50µg/l Arochlor 1254, semi-thin sections of zebrafish testes showed that in the lobule lumen, only few Sp are present, but germ cells in the efferent ducts and spermatocytes are still present in a high number.*

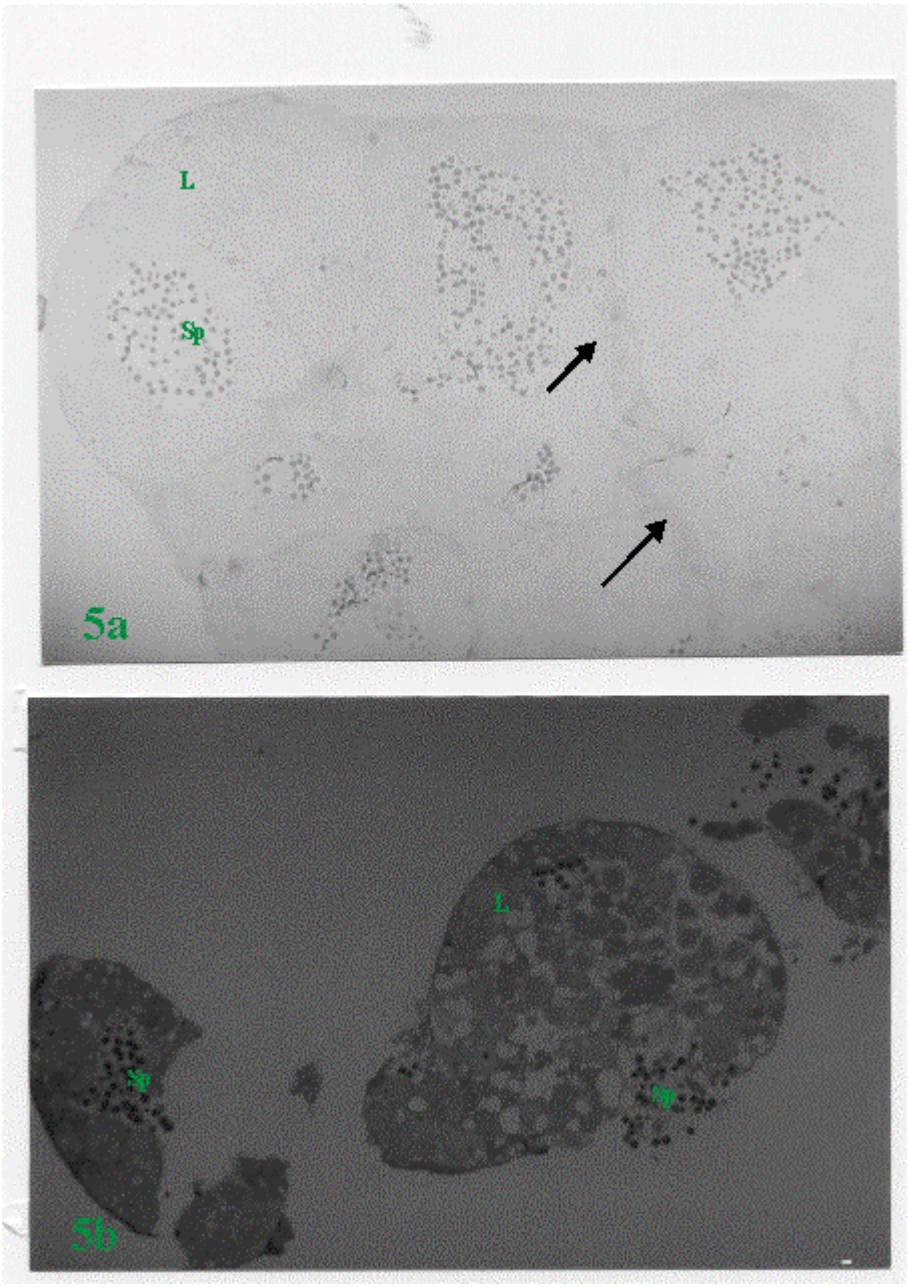


Plate 5a: *After 2 months of exposure to 0.05 µg/l Arochlor 1254, the semi-thin sections of zebrafish testes showed the presence of numerous Sp and efferent duct cells in the lobule lumen. The large lumen is surrounded by the thin walls of the lobules pointing out the enlargement of the interlobular space (arrow).*

Plate 5b: *After 2 months of exposure to 0.5µg/l Arochlor 1254, the semi-thin sections of zebrafish testes showed the lobule lumen releasing mature sperm in a reduced number. But numerous Sg and germ cells are still present in the lumen.*

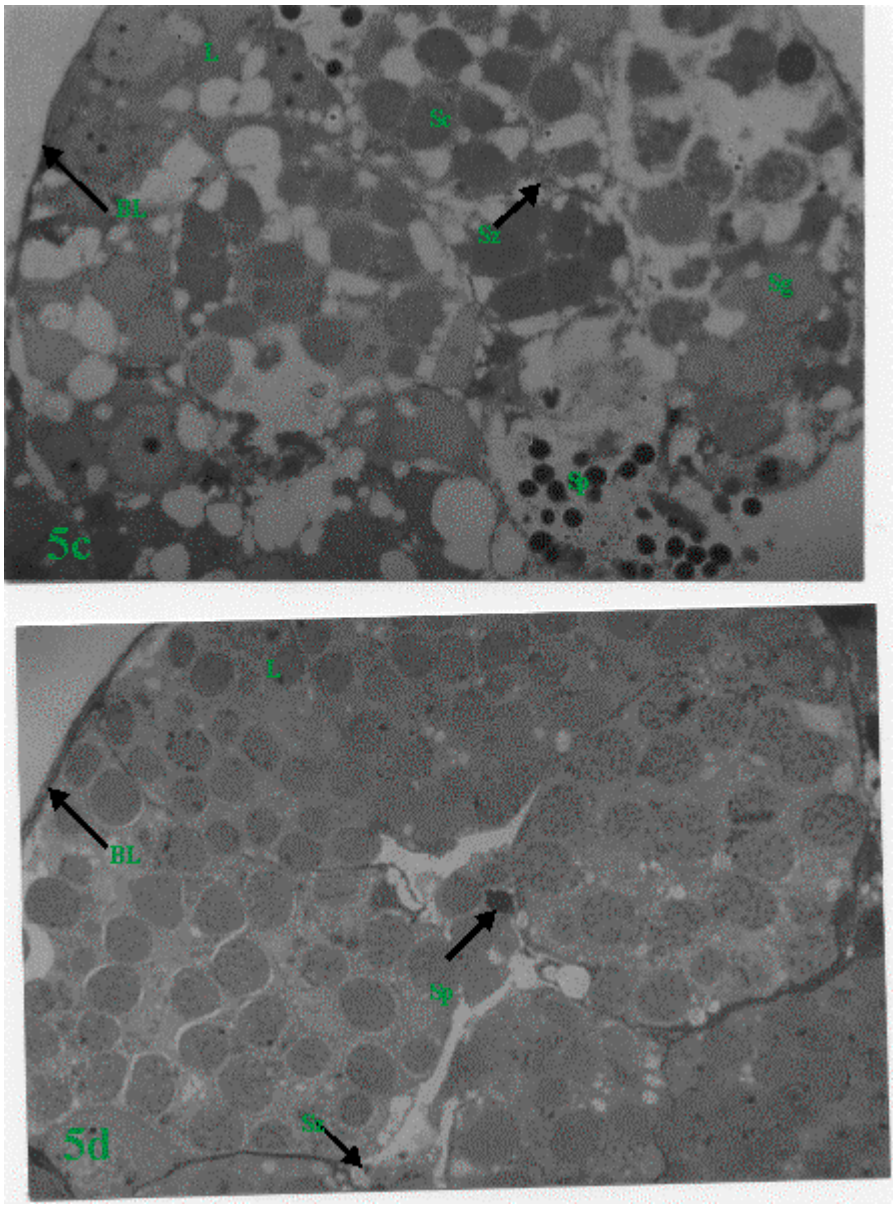


Plate 5c: After 2 months of exposure to 5µg/l Arochlor 1254, the semi-thin sections of zebrafish testes still showed the lumen filled with Sp and germ cells of the efferent duct, but not that much of Sp.

Plate5d: After 2 months of exposure to 50µg/l Arochlor 1254, the semi-thin sections of zebrafish testes clearly showed that later stages of spermatogenesis are not present in a high number in the lobule lumen. The interlobular space of the efferent duct is more pronounced.

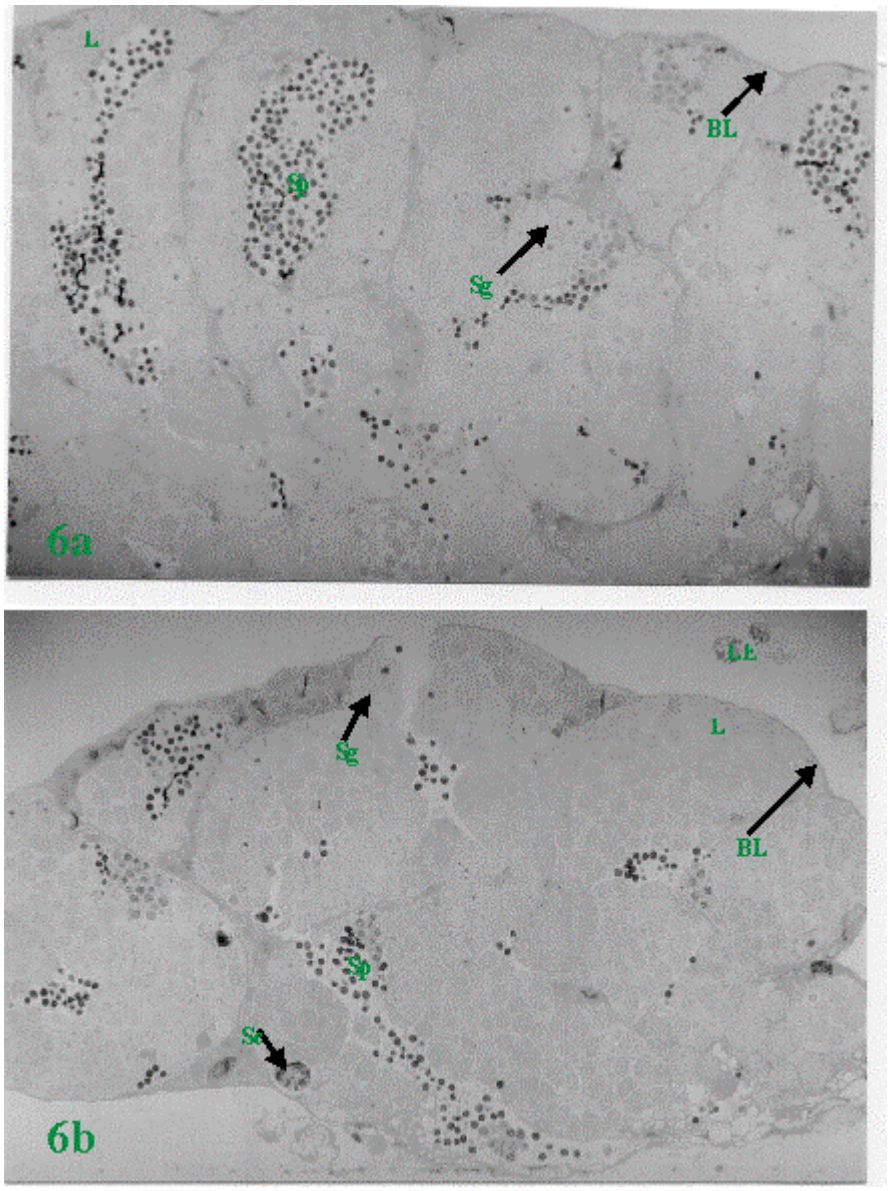


Plate 6a: *After 1 month of exposure to 0.05µg/l of the 1:1 mixture of DDT and Arochlor 1254, the thin-sections of zebrafish testes showed the lumen filled with Sp and germ cells. The number of Sp is not as high as in that of the non contaminated ones. The presence of numerous divisions in the lumen could also be mentioned.*

Plate 6b: *After 1 month of exposure to 0.5 µg/l of the 1:1 mixture of DDT and Arochlor 1254, the thin sections of the zebrafish testes showed that the decrease in the number of Sp in the lumen persists and the enlargement of the interlobular space has started. We could also notice the presence of some Sg during the release of Sp by the the lumen.*

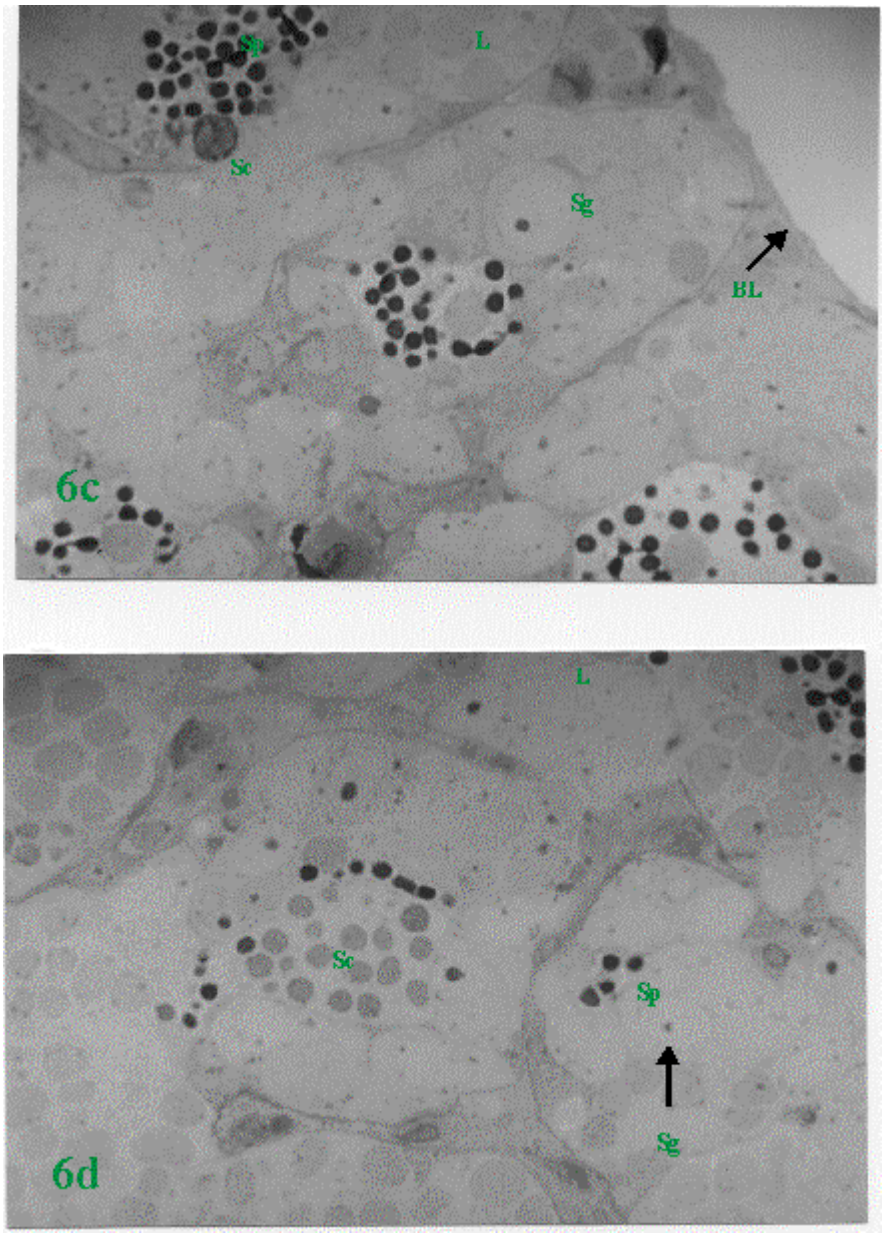


Plate 6c: *After 1 month of exposure to 5µg/l of the 1:1 mixture of DDT and Arochlor 1254, the semi-thin sections of zebrafish testes confirmed the reduction of the Sp content in the lobule lumen and the enlargement of the the interlobular space.*

Plate 6d: *After 1 month of exposure to the 1:1 mixture of DDT and Arochlor 1254, the semi-thin sections of zebrafish showed almost the same reults as on the plate 6c only that the presence of numerous Sc could be mentioned.*

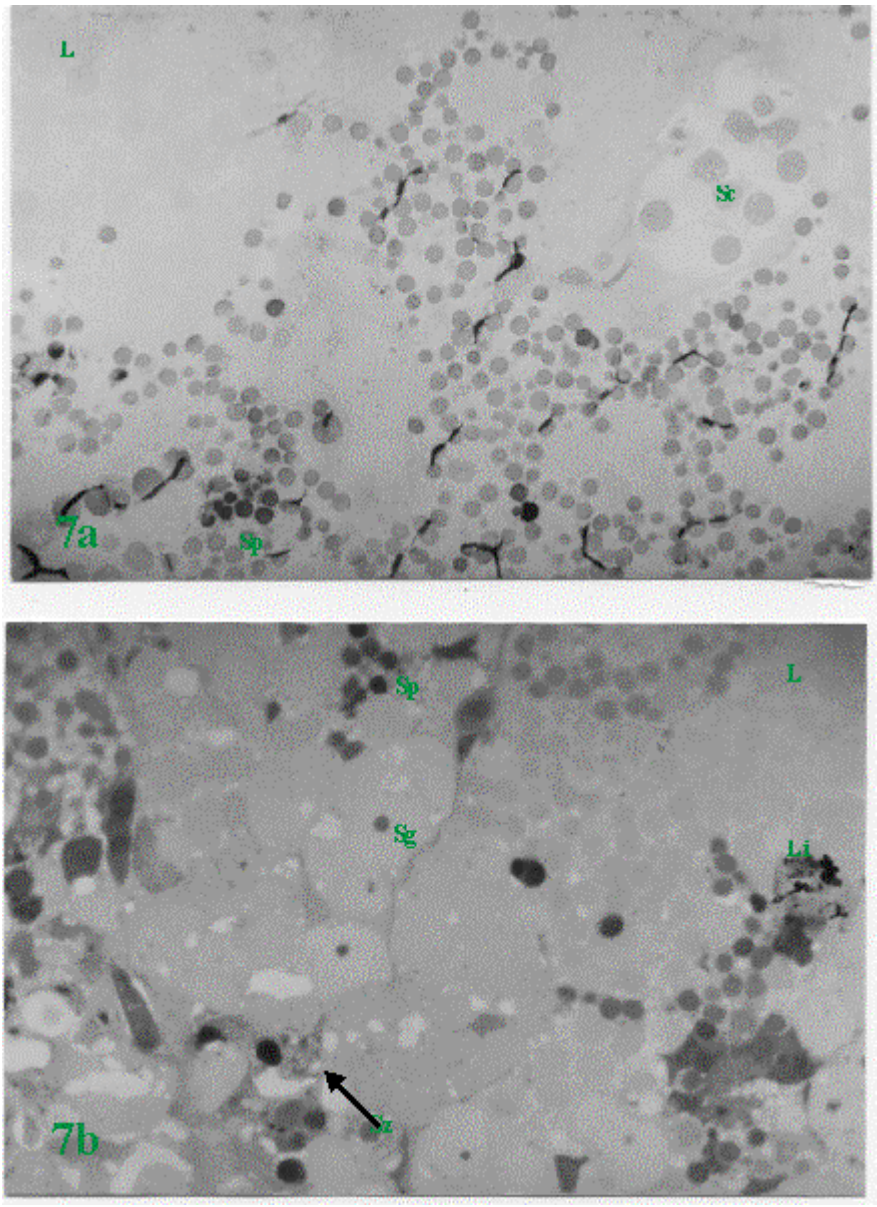


Plate 7a: *After 2 months of exposure to 0.05µg/l of the 1:1 mixture of DDT and Arochlor 1254, the semi-thin sections of the zebrafish testes showed the lobule lumen filled with numerous germ cells of the efferent duct and some Sp.*

Plate 7b: *After 2 months of exposure to 0.5µg/l of the 1:1 mixture of DDT and Arochlor1254, the semi-thin sections of the zebrafish testes showed the reduction of the Sp content in the lumen, the presence of some lipid droplets and the apparition of the lobular space.*

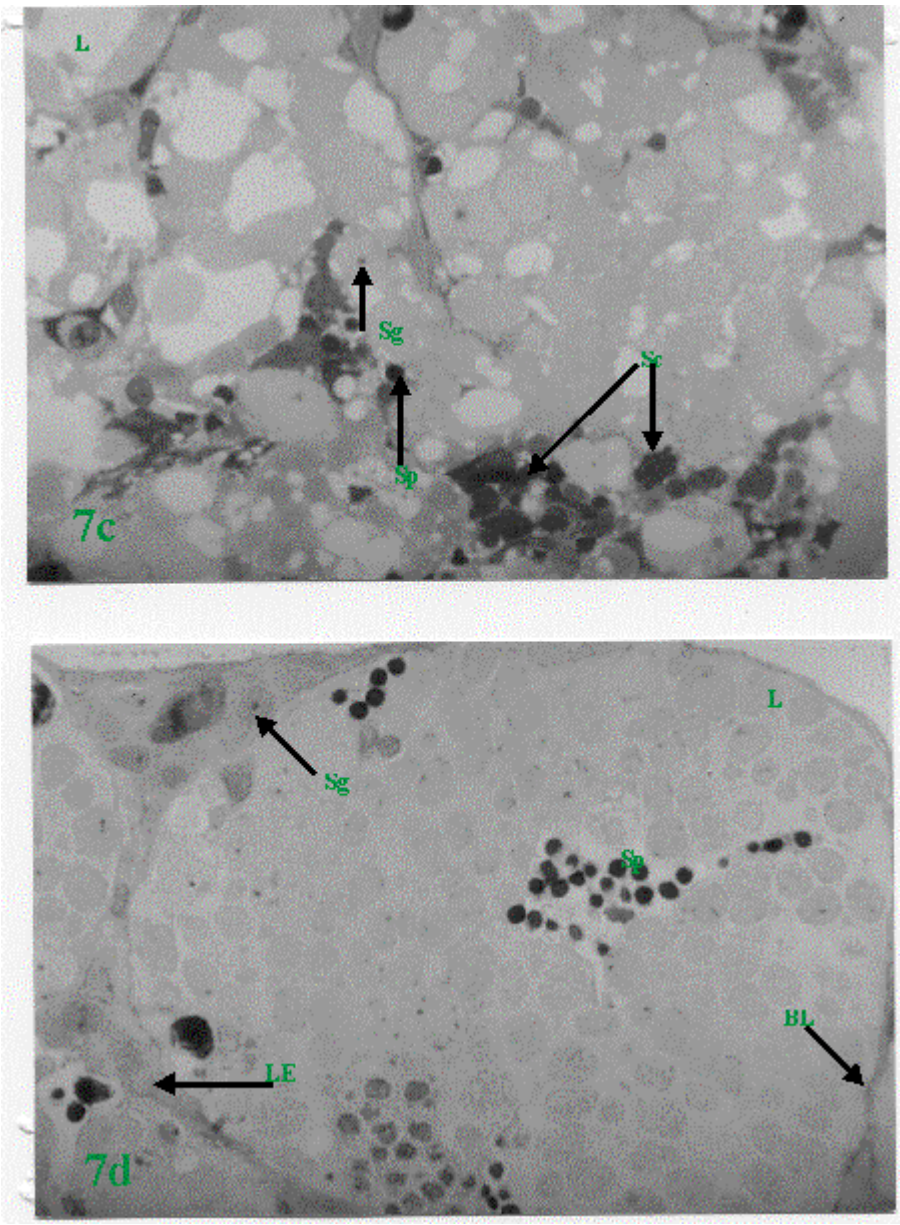


Plate 7c: *After 2 months of exposure to 5µg/l of the 1:1 mixture of DDT and Arochlor1254, the semi-thin sections of the zebrafish testes showed that the decrease of the Sp content in the lumen becomes more significant when compared to that in the case they are either exposed to DDT or Arochlor alone at this very concentration.*

Plate 7d: *After 2 months of exposure to 50µg/l of the 1:1 mixture of DDT and Arochlor 1254, the semi-thin sections of zebrafish testes showed only the presence of few Sp in the lumen. The space between lobular lumen revealed the presence of Sg mixed with LE.*

Electron Microscopy

The following plates show the ultra-thin sections of the zebrafish testes at all the tested concentrations of DDT, Arochlor 1254 and their 1:1 mixture within 2 months of exposure in each case respectively.

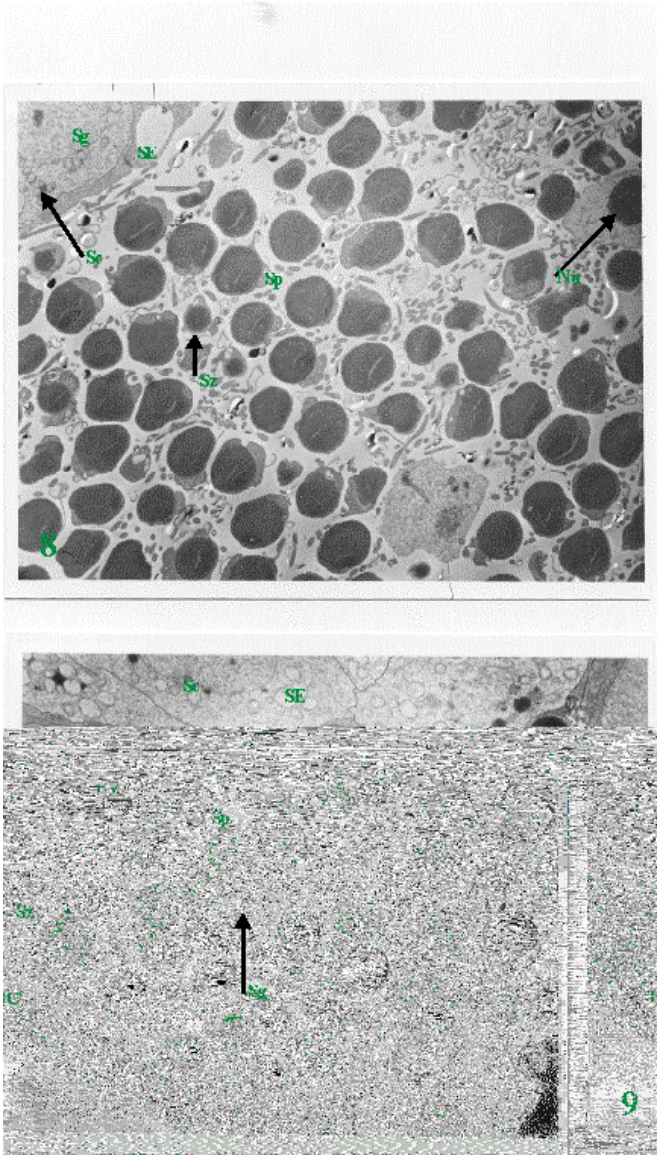


Plate 8: *Ultra-thin sections of the uncontaminated zebrafish (control) testes. The Sp at different developmental stages free in the lumen of a testicular efferent duct. A Sg individually surrounded Sertoli cells (SE) projections. Note the high nucleus to cytoplasm ratio and the cytoplasmic nuage associated with residual bodies (RB).*

Plate 9: Ultra-thin sections of zebrafish tested after 1 month of exposure to 5µg/l DDT. The lumen showed the Sp at different developmental stages. Some cytoplasmic lipids associated in Sertoli cells are associated with a Sg. A prominent blood vessel (BV) is observed and a boundary cell (BC) bordering lobule surface.

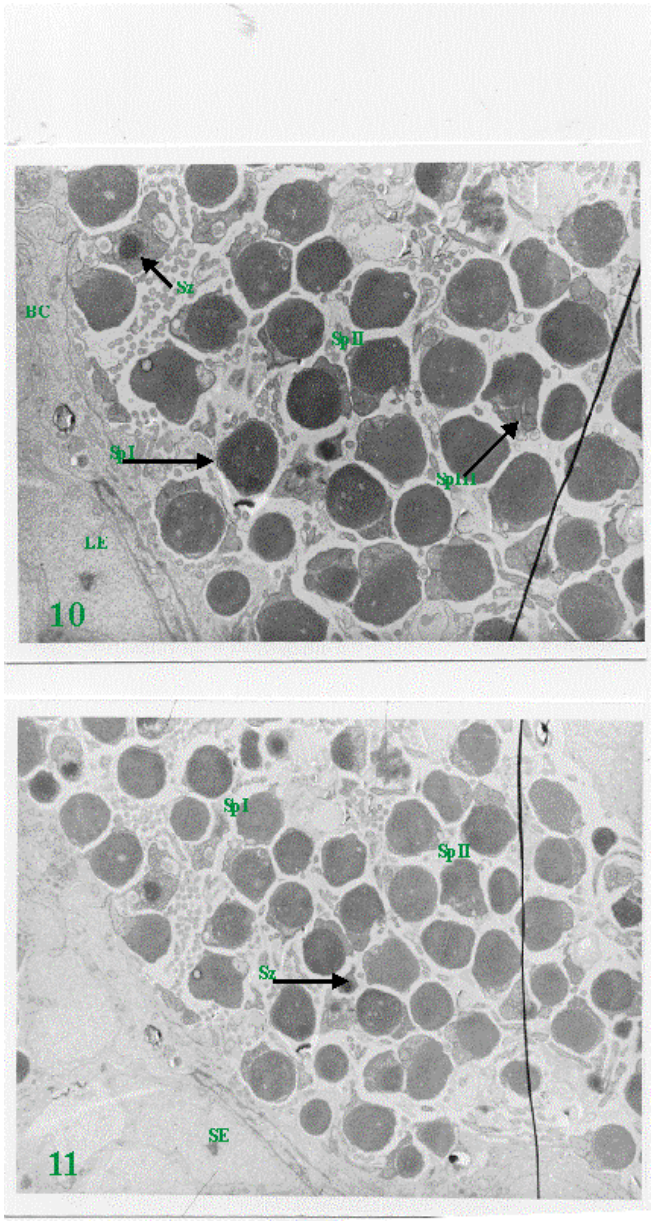


Plate 10**:** *Ultra-thin sections of zebrafish testes after 1 month of exposure to 50µg/l DDT. The late stage of spermatogenesis is mostly constituted by round spermatids I (SpI) and II (SpII) and only few spermatids III (SpIII) and Spermatozoa (Sz).*

Plate11**:** *Ultrathin-sections of zebrafish testes after 2 months of exposure to 5µg/l DDT. The lumen is filled most of SpI and SpII with ripe spermatozoa appearing side by side***.**

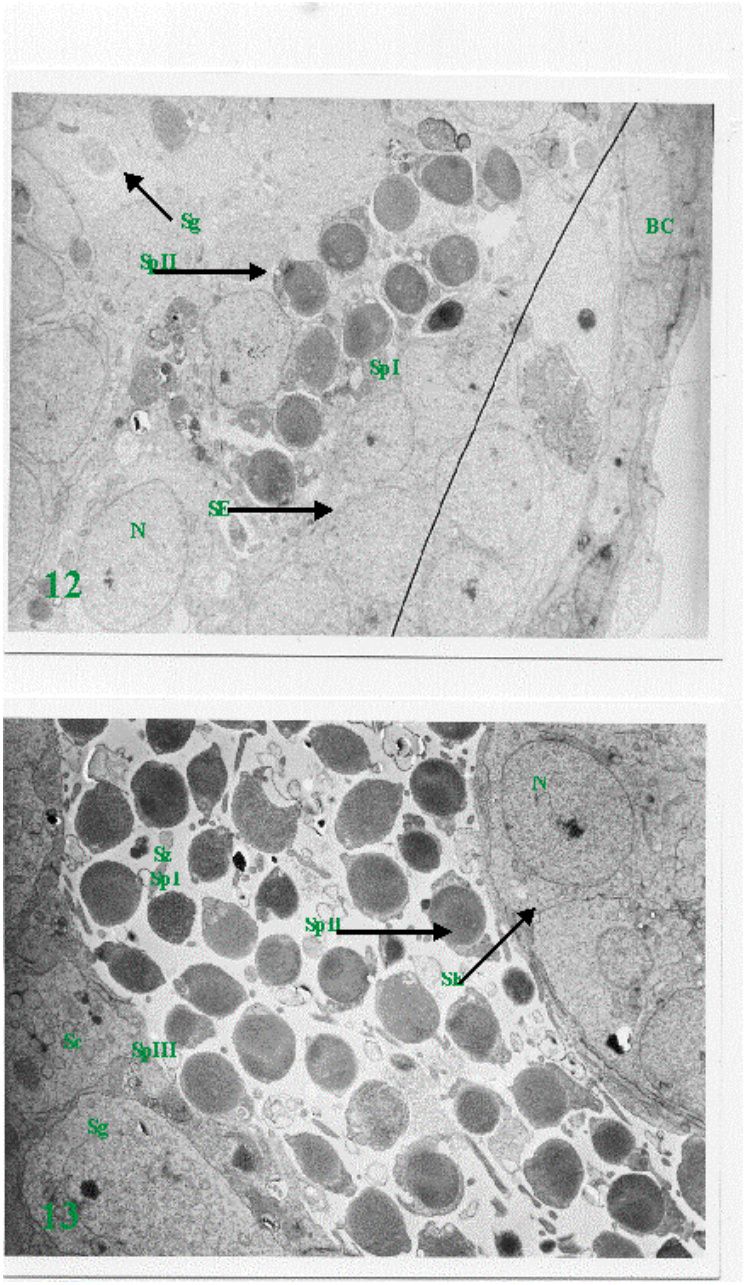
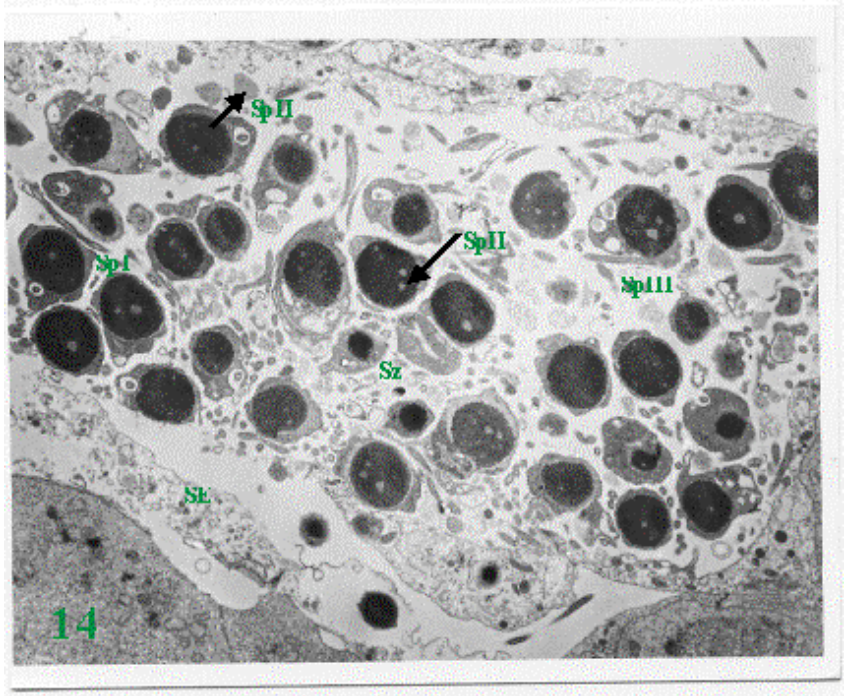


Plate 12: Ultra-thin sections of the zebrafish testes after 2 months of exposure to DDT. Few spermatids at stages I and II are present in the lobule lumen with detail of a Sertoli cell whose main cytoplasmic characteristic is the presence of numerous nucleus.

Plate 13: *Ultra-thin sections of zebrafish tested after 1 month of exposure to 5µg/l A54. Round spermatids at stage I are mostly present in the lumen with Sertoli cells surrounding spermatogones and Dividing spermatocytes. Spermatids III and Spermatozoa are not that much.*



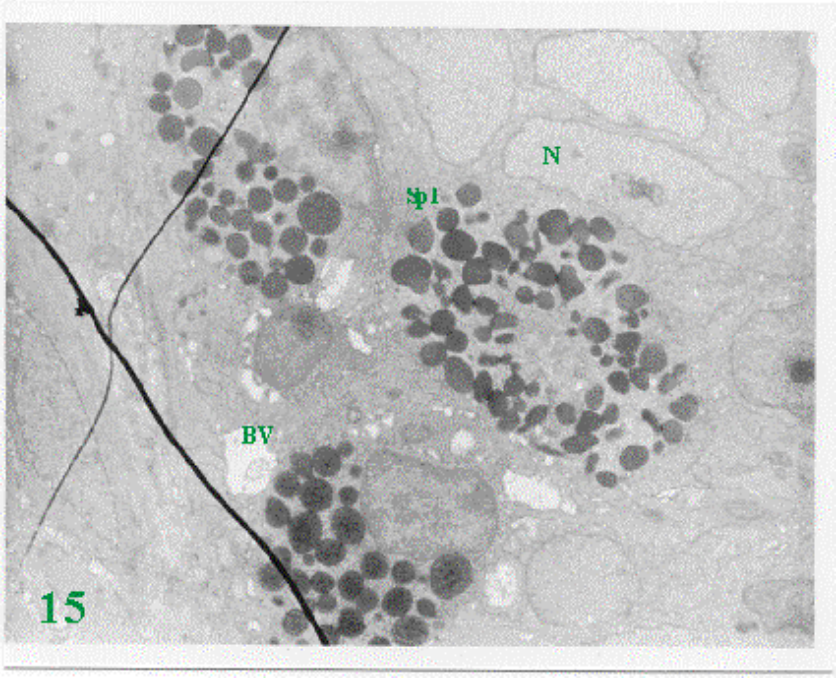


Plate 14: *Ultra-thin sections of zebrafish testes after 1 month of exposure to 50µg/l A54.*
The lumen is still mostly filled with round spermatidsI although from side to side are the spermatoa present
Plate 15: Ultra-thin sections of zebrafish testes after 2 months of exposure to 5 µg/l A54 clearly showing in the lumen the presence only of round spermatids at primary stage and prominent blood vessels (BV).

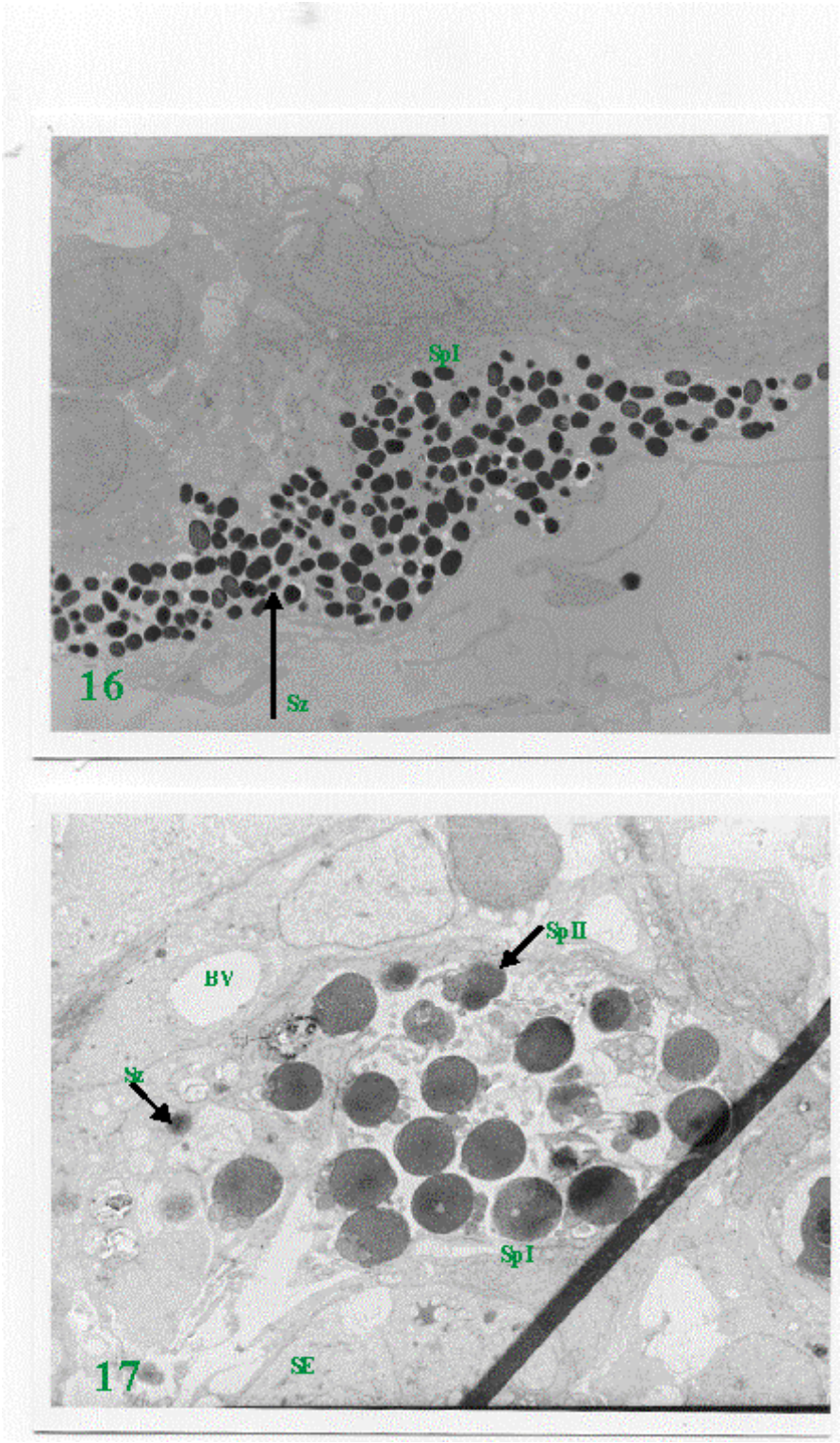


Plate 16: *Ultra-thin sections of zebrafish testes after 2 months of exposure to A54. Only round spermatidsI are visible in the lumen excluding their developmental stages.*

Plate 17: Ultra-thin sections of zebrafish testes exposed to 5µg/l of the 1:1 mixture of A54 and DDT. The lobule lumen is filled with few Sp and mostly the SpI and SpII.

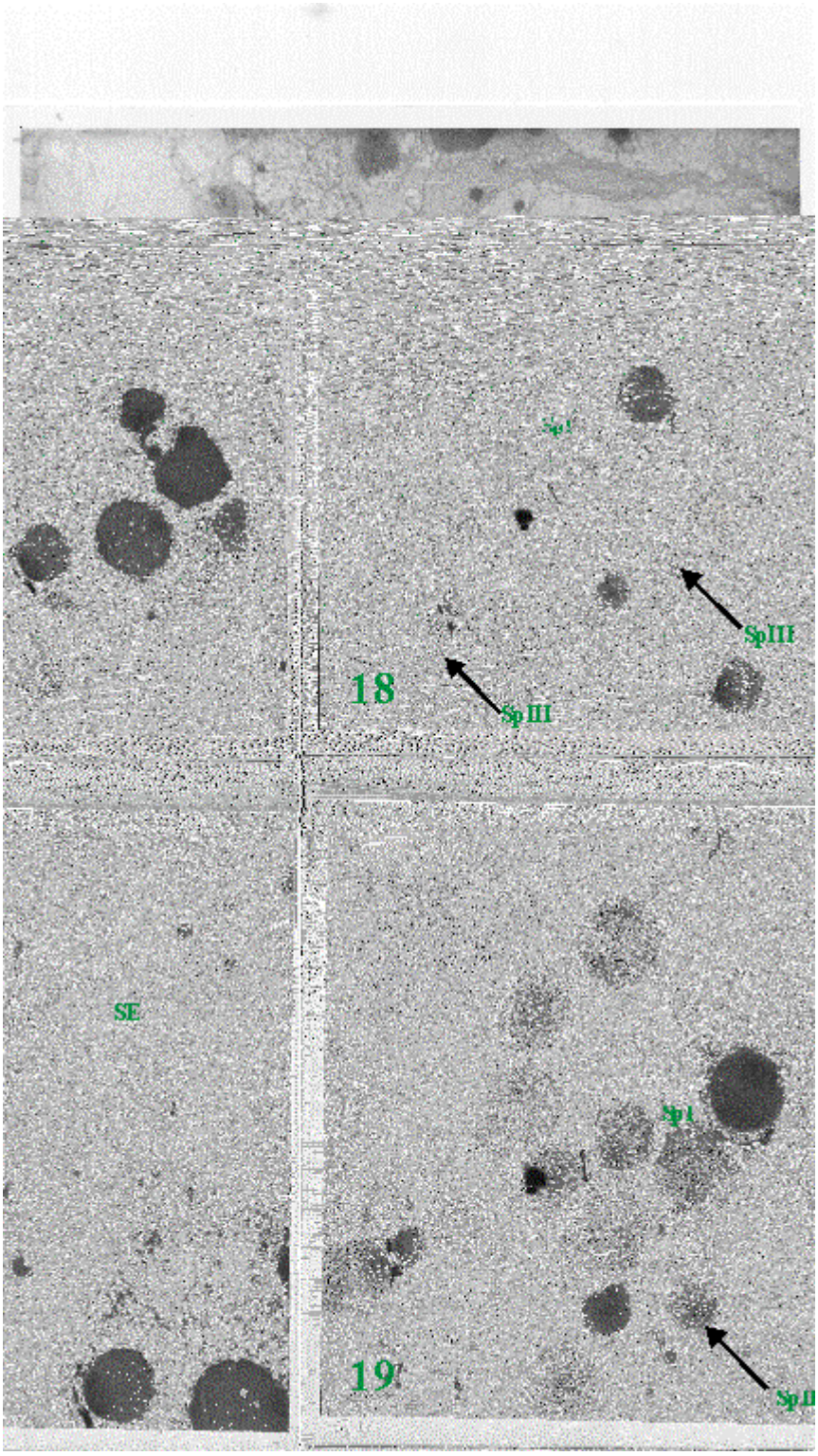


Plate 18: *Ultra-thin sections of the zebrafish testes after 1 month of exposure to 50µg/l of the 1:1 mixture of DDT and A54. Some few spermatids , mostly at the primary stage are present in the lumen.*

Plate 19: Ultra-thin sections of the zebrafish testes after 2 months of exposure to 5µg/l of the mixture of DDT and A54. More reduced number of spermatids mostly at primary and secondary stages are present in the lumen.

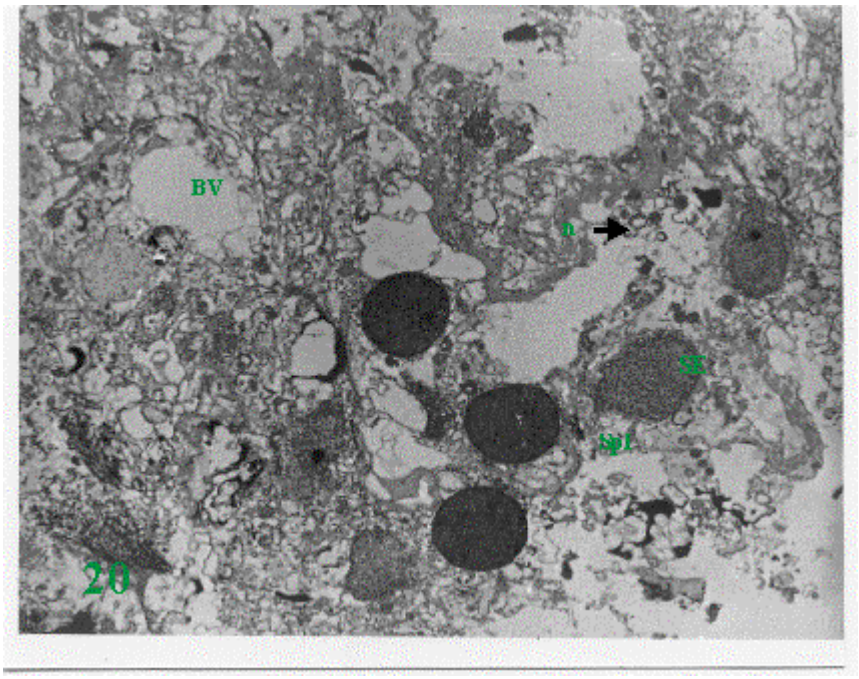


Plate 20: *Ultra-thin sections of zebrafish testes after 2 months of exposure to 50µg/l of the mixture of DDT and A54. There are very few round spermatids at primary stage mixed with prominent blood vessels and nuages (n) in a cytoplasm with a cloudy aspect.*

4) Discussion

4.1) Toxicity tests

4.1.1) Toxicity test with DDT

The NOEC at the tested concentration of 0.05µg/l within 21 days of exposure agrees with the report of Mayer and Ellersieck (1986) on the 96h-LC50 which ranged from 1.5 to 56µg/l for large mouth bass and guppy respectively. This simply means, according to the size of fish we used, that the concentrations of DDT below 1.5µg/l cannot have any effect on them. However we reported a mortality at the concentration of 0.5µg/l in 21 days which is still a concentration far away to reach the 21 days-LC50.

The mortality started to become significant at the concentrations 5µg/l and above. At the concentration of 50µg/l, we could remark that the 21 days-LC50 was already reached on the seventh day after exposure because half of the population was already died.

At the tested concentration of 500µg/l, more than 50% of the fish population already succumbed after 12 hours of exposure and therefore the LC50 could also at this very time be established. It could then be proposed that, this tested concentration was too high and too toxic to the fish specimens and seemed to have reacted without taking in account the size of the fish.

The frequency of the mortality after the exposure of fish to DDT was not regular and uniform. At the beginning, the mortality recorded remained constant within a long period and became low afterwards. It looked like the fish acclimatized after a period of time with the new rearing area. This result could also be due to the fact that smaller fish first uptake DDT and the bigger resist for some time, which agrees with Murphy (1971), who reported that, a range in weight of mosquitoesfish between 70 and 1000mg led to a four-fold difference between the smallest and the largest fish in DDT uptake from water over 48 hours. The 21 days-LC50 can be ranged between 5 and 50µg/l. This LC50 is bigger than 5µg/l and smaller than 50µg/l.

For the short term exposure, at all the tested concentrations, mortality commenced only 6 hours after exposure. This could have been correlated to the temperature which should have increased enough leading also to an increase in DDT uptake as pointed out Reinert in 1974. Within 96 h at the lower tested levels , the uptake of DDT is negligeable because the fish started to succumb at the concentration of 5µg/l only after 48 h and in a low rate.

4.1.2) Toxicity test with Arochlor 1254

Arochlor1254 showed almost the similar toxic effects with DDT. But the 100% mortality was recorded at the concentration 500µg/l only after 12 days of exposure. Per consequent no estimation on the 96h-LC50 could be made due to the half population size which did not succumb in any of the case before this very time. After 21 days, the lowest rate of mortality recorded was 10% at 0.5µg/l and on the 20^{th} day after exposure. The frequency of mortality after exposure was not also regular and uniform. The tested chemical showed similarities in its effect with DDT, but seems to be more tolerated than the latter by the test organisms. It could then be proposed that toxicity effect of fish with Arochlor 1254 slighter than that with DDT at the same tested concentrations.

The 21 days LC50 can be ranged between 50 and 500µg/l. This LC50 is bigger than 50µg/l and lower than 500µg/l.

For the short term exposure, no mortality was recorded at the three lowest concentrations and was only 20 and 40% at 50µg/l and 500µg/l respectively after 96h of exposure. Arochlor 1254 had demonstrated that it can persist and accumulate in the environment and potentially causing toxic effects as reported Kutz et al in 1991.

The uptake before the fourth was slower than that in the case of DDT, suggesting that a steady state has already been reached. Exposure of fish for a further 96h in the test chambers supported this theory. Further mortalities recorded were just a proof that, the elimination of the chemical was slow and still influenced the test organisms afterwards.

4.1.3) Toxicity test with the 1:1 mixture of DDT and Arochlor 1254

In contrast to DDT and Arochlor 1254, the fish were more sensitive to the 1:1 mixture of both chemicals at the same tested levels which consequently provoked earlier their death. At the lowest concentration, the long term exposure ended with a 10% mortality while at the highest tested concentration, after 6 hours of exposure, the rate of mortality was already up to 70 %. At the tested concentration of 5µg/l after the 17^{th} day, an LC50 could be already established. The 1:1 mixture of the pesticides DDT and Arochlor 1254 produced a higher effect on fish than when they are present alone. This observation agrees with the one of Arnold et al (1996) who reported that a range of chemicals could act synergically to bind and activate the human estrogen receptor (hER). We could then proposed in the case of the

present experiment that, there is a synergism between DDT and Arochlor 1254 which is likely to present a major fish health concern.

4.2) Accumulation of DDT and Arochlor 1254 in zebrafish

During the bioaccumulation test in a static condition with DDT and Arochlor 1254, the evaluation of the BCFs was a proof that the zebrafish really picked up and accumulated the pesticides. The results obtained agreed with those expected by the OECD guidelines for the testing of chemicals, since the reduction in the level of the tested chemicals in the blank aquaria were not that significant.

The BCF values with DDT as tested chemical were higher than those of when Arochlor 1254 was the tested chemical, which led us to conclude that DDT was readily faster taken up by the zebrafish than Arochlor 1254. It could then be established that the pesticides exhibit effects on the zebrafish.

The fat content during exposure of fish to the chemicals at all the tested concentrations did not show any significant difference in their levels. This simply means although the pesticides are known to accumulate more in the fatty tissues, they do not affect their levels in contaminated fish within 8 days. This observation agrees with some reports based on the correlation of fat content and the level of pesticide for instance that of Wallace and Hulmes (1977) who found a low variation in fat content between the different fishing grounds and its increase with length for cornish mackerel, as well as that of Stronkhorst (1992) supporting the fact that, there tends to be an increase of dieldrin and pp'-DDE content with length of fish but this could also be associated to higher fat content of larger fish. More interest should be put on the seasonal variation of the fat content in fish, accumulation by age or length where the informations are still rare as already pointed out Kamman in 1990 and Miller in 1994.

The pesticides accumulated in fish within 8 days, but this observation was not estimated with regard to the weight of fish since Westernhagen et al (1995) have already reported that, in general there is no correlation between accumulation of chlorinated compounds and age or size. It was then observed that the more the pesticides are present in a big quantity in the medium, the more they accumulate in fish. That could be the reason why the BCF8days values were found increasing with increased chemical levels.

The results tended to agree with those of Fox et al who found that the BCF values increase with the degree of chlorination from dichlorobiphenyl 9 to hexachlorobiphenyl 169 and the heptachlorobiphenyl 185.

The tested concentration of 0.05µg/l was not a concentration high enough to evaluate the BCF in fish within 8 days of exposure because with this level, the pesticides were not more detectable in the medium containing the test organisms.

In both cases, at all the tested concentrations, no equilibrum was reached within 8 days, and per consequent, it could be proposed that the chemicals don't reach their stable or constant concentration of accumulation in a given static aquatic environment within 8 days. Longer bioaccumulation tests could be therefore recommended in order to fulfil such investigations. The organochlorine distribution in the aquatic environment can be understood by considering the distinctive physiochemical properties of low water but high lipid solubility and relative inertness of the compounds. In its simplest form an equilibrum is hypothesised to develop between concentrations in water and lipid phase of marine organisms as pointed out Falkner and Simonis in 1982, and Pruell et al in 1993. As The BCF values have been evaluated, we could be able to establish the potential hazard of the chemicals and to know whether the chemicals could be resorbed and if so, at which amounts they could have been taken up by the fish which is an indispensable precondition. After uptake by an organism, the amount of xenobiotic compounds which could not be excreted will persist in the body and will be accumulated. Harmful effects could only be induced, when the substance has been accumulated in sufficiently high concentrations at the site of toxic action. We also investigated some aspects of the life cycle which could be influenced by this accumulation.

4.3) Duration of the LCS and length of fish emerged at all the DDT, Arochlor 1254 and their 1:1 mixture tested concentrations.

To assess the chronic effects of xenobiotics on fish, established test systems such as acute or prolonged toxicity tests are not adequate because it is not possible to extrapolate from these data to long term effects. So it is necessary to develop new test systems which are able to detect chronic effects. One of the most important effects on fish populations is the impairment of reproduction as reported Ensenbach and Nagel in 1997. However, by investigating many of these parameters, early life stage tests have been shown to be inadequate, a full life test should be carried out.

All the tested concentrations of DDT, Arochlor 1254 and their 1:1 mixture had an effect on the hatchability, growth and duration of the LCS in such a way that in none of the case could the NOEC be established.

At the tested concentration 500µg/l, the lack of hatchability rendered impossible the evaluation of the parameters which were wished to be investigated in the next stages of the L.C. This situation has brought us to conclude that both pesticides and their 1:1 mixture ranging around this level are too toxic to the ELS of zebrafish.

The rate of hatchability was reduced in 6, 27, 43 and 62% for DDT, in 3, 16, 28 and 35% for Arochlor 1254 and in 7, 37, 52 and 71% for their 1:1 mixture at 0.05, 0.5, 5 and 50µg/l of each respectively. This reduction in the rate of hatchability is found to be a factor correlated to the amount of the initial tested concentrations, because in the case of our study, it has increased with increased tested levels of the chemicals.

From day 109, glass dishes installed in the aquaria to assess whether spawning had already begun were containing eggs. The quantity of produced eggs laid within the range known from the literature. Nagel (1988) ascertained variations from 40 up to 160 eggs and Bresh et al (1990) published a mean value of 100 eggs per female per day.

On the concentrations of 0.05 and 0.5µg/l of the tested chemicals, no negative effect on egg production could be detected. A significant effect on egg production was however found at the highest xenobiotic concentration. The number of eggs decreased in more than 50% for DDT and the mixture DDT and Arochlor 1254 and only in 35% for Arochlor1254. Approximately 16 days after, the egg production had totally stopped at the highest concentrations. This effect was linked to the bad condition of fish and behavioural changes. No courtship of the males occurred. Normally, healthy fish react very quickly if food reached the bottom of the basin. It could be proposed that the effect of the individual chemical DDT and the xenobiotic mixture caused a reduction in the rate of hatchability of zebrafish. This reduction of the rate of hatchability was also observed in the F-II generation and in fact, the effect of the pesticides and their 1:1 mixture was widespread to the next stages of the LC. In the F-I generation a reduction in the survival rate of the offsprings was also mentioned. This reduction was in 36, 56, and 88% for DDT, in 30, 37 and 70% for Arochlor 1254 and in 50, 67 and 92% for their 1:1 mixture at 0.05, 0.5 and 5µg/l respectively. In all the cases, the juveniles couldn't emerge up to the F-II generation at the initial dosed concentration of 50µg/l. These results were a reason to question about the report of Ensenbach and Nagel in 1997 in which they stated that the xenobiotics Lindane and 3,4-dichloroaniline had no effects on survival rates of zebrafish and that the death of non surviving fish was connected with a

nutritional change. They then emphasized their report with the fact that the yolk sac of larvae was consumed and larvae have to search food in aquaria. When larvae were not able to seek their food, they died by starvation at the end of the second week. But in the present experiment, the survival rate was found correlated to the level of the tested chemicals and the exposure time.

According to the report of Mattig et al (1997) in which they confirmed that the main factor determining the total DDT concentrations is the fat content, we could notice that the high rate of mortality and disorders recorded during the LCS of fish emerged could be due to the fact that during the growth of of juveniles, their fat content has increased and consequently has allowed them to accumulate more chemicals. It could be then proposed that the mortality rate increased with increased levels of the tested chemicals and was higher in treated basins than in controls. The length of juvenile fish was significantly reduced at the concentrations of 5 and 50µg/l DDT and Arochlor 1254 respectively. Comparing the early life stage tests of F-I and F-II generations, the length of juvenile fish emerged could be mentioned as additional effect which appeared. So the raising of parent fish in the xenobiotic mixture influenced the length of their young and this with higher levels of the chemicals.

On the other hand, the growth response of the juveniles has been found delayed for some days, since the duration of the larval development lasted longer than expected when compared to that of the uncontaminated ones and therefore it was also found correlated to the initial tested levels of the chemicals up to the F-II generation. It has been assumed that, the chemicals accumulated in eggs and thereafter widespread to the juvenile fish emerged, which demonstrated how sensitive was the ELS to them. This result agrees with the report of Westernhagen et al (1995) which approved that the content of chlorinated compounds in the ovaries and livers of various fish species are found in general not correlated to the age or size.

In general it could be proposed that the accumulation of DDT and Arochlor 1254 in eggs is widespread to the offsprings by disturbing the normal expected duration of their LCS, their rate of of hatchability at the beginning of the next generation., their population size as well as their growth. These investigations could be emphasized by the report of Welsch (1998) in which he mentioned that there have been data published recently (Nagel et al, 1997, Vom Saal et al, 1997) confirming that the time of prenatal development and differenciation of sexual organs are for endogen hormons the most sensitive phases. Very comparable effects on synthetic exogen hormons (eg. Diethylstilbestrol, Vom Saal, 1997) and the ones as “ environmental hormons” suspicious substance Bisphenol A (Nagel et al, 1997). Bisphenol A is an important constituent of polycarbonate plastic materials and epoxy resins with further

spread in human habitat. This substance has already been checked up with conventional methods of the toxicological reproduction at found only at the extrem high doses to produce significant estrogenic effects.

From these observations in animal researches , could result many important questions to the toxicology. For eg , are the endocrine active substances including the exogen “environmental hormone” totally different from other chemical substances? Should the toxicologists faced to the pressing becoming problem which is the evolution of human health risiko to the environmental hormone, surround all their ways to think on the dose effects? This could mean that the experiment with animals should be concentrated on the highest sensitive phase of prenatal development and sexual differenciation. This position will be emphasized by Dr Theo Colburn (Author of “Our futur in danger”). Afterwards, “environmental hormons” which are biologically active at concentrations under the usual dosages in the evaluation of safety are situated in conventional toxicology. Gulden et al, in 1997 have reported after a work carried out in their institute that, it has been shown that a big number of synthetic substances could also have reacted as endocrine. But the estrogenic effects are the most represented when the antiestrogenic and specially androgenic substances are in small number. He also classified the pesticide DDT and the persitent organo halogens PCBs as with widespread distribution reported to have reproductive and endocrine disrupting effects. The parameters investigated in the present study were among the 5 new research fields pointed out by Dr Hasso Seibert of the Institut für Toxicology, University of Kiel in 1998. As example of one of the five fields, was the development and standardisation of strategic tests to assess chemicals and environmental samples on the endocrine activity.

The report of Schettler in 1998 could help us also to explain the results obtained in this study by defining the endocrine disruptors. The endocrine system utilizes circulating hormones to help integrate the functions of individual organs and the nervous and immune systems.

Complex interactions among these integrating systems are responsible for control, regulation and maintenance of homeostasis, reproduction, development and behaviour. Hormones exert their effects through binding to a specific receptor. In turn the activated receptor initiates a cascade of biochemical events. An endocrine disruptor is an agent that produces reversible or irreversible biological effects in individuals or populations by interferring with hormone function. They may temporarly or permanently alter feedback loops involving the brain, pituitary, gonads, thyroid gland, or other organs. Their action is not limited to the receptor binding.

Timing, duration and amount of exposure are each important determinant of the outcome. There are windows of vulnerability during fetal development in which small exposures to endocrine disruptors may have profound effects not observed in adults.

For instance, studies of the intrauterine position of mice during fetal development show that, slight fluctuations of steroid hormone levels influence genital morphology, timing of puberty, sexual attractiveness, sexual behaviour, aggressiveness, and activity level of offsprings (Schettler, 1998). Also in mice, a single small maternal dioxin exposure on day 15 of pregnancy causes delayed testicular descent, impaired sperm production, abnormal hormon levels, and altered behavior in male offspring. It has been also reported that the dose response curves for endocrine disruptors sometimes vary from those to which we are accustomed.

Male mice exposed in utero to DES, DDT, or methoxychlor, anorganochlorine pesticide, showed aggressive behavioral changes. The unusual dose-response is also seen in rats exposed to PCB's in utero. Very small doses have no effect on litter size or physical development but caused impaired learning and hyperactivity in offspring. Higher exposure levels have no impact on activity, but litter size is decreased and neuromuscular maturation delayed.

Male and juvenile fish exposed to Akylphenol polyethoxylates, estrogenic substances commonly found in sewage effluent, produce two proteins normally produced only by female fish a yolk protein, vitellogenin, and an eggshell protein. We do not yet know if these chemicals have impacts on the reproductive success of these fish. Does that mean that as a signal that may represent important but incompletely understood effects?

The list of known endocrine disruptors varies in length depending on individual interpretations of the evidence. Approximately 40 pesticides are on the list, including herbicides, insecticides, and fungicides, many of which are in widespread commercial use. They are joined by a series of industrial chemicals, some of which, like alkylphenols and phtalates are produced in enormous quantities for multiple uses and found throughout the world's ecosystems.

Subtle effects on neurological development and behavior are quite difficult to study. Adverse effects which are delayed, sometimes by decades in human, are difficult to link with early exposures. And some such as the effects of exposures on skeletal development and maintenance, have been studied very little.

Sources of information include laboratory, wildlife and human epidemiological studies. Population-wide trends in certain diseases and biological measurements add important data. Acting through the Ah receptor, in laboratory animals, in utero exposure to some dioxins and

PCB's reduce fertility, testis weight, and alter hormone levels, interfering with the functioning of feedback loops which normally correct these alterations. Similar hormone level changes are seen in occupationally exposed humans.

Some dioxins and PCBs alter thyroid hormone function, acting through several mechanisms, including competition for binding to the thyroid hormone receptor or competition for binding to transport proteins, particularly transthyretin. This may well explain neurological abnormalities seen in the offsprings of animals exposed during pregnancy. Wild life study has demonstrated a relationship between exposures to endocrine disrupting substances and abnormal thyroid function, sex alteration, poor hatching success, decreased fertility and growth, and altered behavior.

Female fish downstream from pulp and pafer mills have developed male sex organs and have altered behavior. Males were hypermasculinized, showing aggressive mating behavior. The agents responsible have not been identified with certainty, but some suspect that they are pulp-mill chemicals which are converted to androgenic

A common theme that runs through many of the wildlife studies is a lack of accurate exposure assessment and exposure to co-occurring mixtures of contaminants. Many of these are ecological or cross-sectional studies in which the adverse effects and mixture of contaminants are noted concurrently. To the extent that observations in the field can be combined with laboratory studies, a better understanding of precise cause-and-effect and dose response relationship will emerge.

All the disorders happened on the reproduction during the exposure of zebrafish to DDT and Arochlor 1254 could be assumed as the epidemiological links to early hormone exposure including the following observations:

-undescended testes (or cryptorchidism) is a major risk factor for developing testicular cancer. Fetal estrogen exposure is a known cause of undescended testes.

-First born males have a higher incidence of testicular cancer and first pregnancies have higher early estrogen levels than subsequent pregnancies.

-Maternal overweight is a risk factor for male-offspring testicular cancer, and overweight is associated with lower levels of sex hormon binding globulin.

These disorders are more pronounced in the case of their 1:1 mixtures, showing once more that both pesticides could act synergically with more efficient effect.

Comparing with the results of some similar works in the case of men for instance, these disorders recorded during the investigation of the LC in our study could be due to a testicular cancer and maldescent (Giwercmann and Skakkebaek, 1992), to urethral abnormalities

(Hypospadias) (Giwercmann and Skakkebaek, 1992), or finally to striking drop in semen volume and sperm counts in normal adults (Carlsen et al, 1992).

All these observations could be of great interest regarding the reports of Kamman et al (1990) and Miller (1994) in which they mentioned the rarety of the informations about the contamination level of fish in relation to the spatial distribution.

Schäfers in 1991 and Nagel in 1994 hoped that complete life cycle tests with zebrafish could be shown to be useful tools in fish toxicology. But they found that there are still some remaining problems. They finally assumed that there has been an observed effect within a LC; they are now able to assess the consequences of this effect on a population of test fish. With reference to the present study, it could be then proposed that, LCS duration, length and reproduction could be established as consequences of toxicity of DDT, Arochlor 1254 and their 1:1 mixture to zebrafish. The partial life cycle developed by Bresh et al (1982) favoured by a group working under the auspices of German Standard Institute (DIN) which rejected the concept of complete LC is still questionable because of the additional informations which could be obtained only by investigating the complete LC or in some cases, the next generation even of a given fish population.

A strong mechanistic case can be made to explain how exposure to ELS and juvenile fish to the chemicals could disturb the normal expected duration of the LCS, the population size, the growth and life span up to the ELS of the F-II generation as it was the case in the present study. Moreover exposure to the chemicals during the experimental period led to an increase of reproductive disorders, resulting in an important reduction of the rate of hatchability and adult survival in the F-II generation. These results could bring us to question on the quality and quantity of eggs and sperm released by the tested organisms after their sexual maturity. The suggested mechanism whereby chemical exposure could induce the parameters assessed, could be offered as hypothesis on which to focus discussion and research.

Investigations on the toxicity of chemicals to fish should be preferably performed using the complete LC tests because some side effects could be detectable only at the end of the cycle.

4.4) Sperm counts

A series of studies in Europe, the UK, and the US indicate that there has been probably a substancial decline in human sperm counts over the past 40 years. Inherent study problems such as age, period of abstinence from sexual activity, and individual and geographical

variability make this challenging to investigate, but the weight of evidence indicates a significant decline.

A recently published autopsy study of men who died of various causes unrelated to their reproductive tract compared results from 1991 with those obtained from a similar population of men in 1981, corrected for age and various other medical and social factors.

In this study, the percentage of subjects with normal spermatogenesis and microscopic exam of their testes declined from 56% to 27% over the ten years period. Complete spermatogenic arrest increased from 8% to 20% over the same period.

Our experiment showed that the male zebrafish (*B.rerio*) usually reacted to the the introduction of a ripe female by courting and leading her closer to its anal region. Early in the courtship sequence, the male always turned and strocked his anal –urogenital region towards the area where was the female, repeating this behaviour several times even before the latter started to react. This was a positive observation because it agrees with what has been described in the report of Marconatto et al (1996) on the sperm release in three gobiid fishes (Teleostei, gobiidae).

During the mating events, the sperm released by male zebrafish increased with time after the introduction of ripe female and no sperm was found in water samples before the introduction of the latter, agreing also with the finding of Marconatto et al (1996). The different sperm concentrations found at each of the tested concentrations and the statistical test performed showed firstly that the accumulated chemicals which consequently affected the reproductive tract by reducing the number of sperm released by the male, and secondly that the number of sperm released is influenced by the duration of the exposure of male fish to the chemicals and their 1:1 mixture.

The use of ANOVA to compare the sperm concentrations after each period of sample collection, showed that the values of the F-ratio increased as the fish remained longer exposed to the pesticides. These values of the F-ratio were also found increasing while comparing the sperm concentrations obtained at the same tested concentrations but with different exposure time in all the cases. It could then be proposed that long term exposure of male zebrafish to DDT, Arochlor1254 and their 1:1 mixture influenced the number of sperm released during the mating events which could be assumed to be a reduction of the semen volume. This proposition could be of great interest to the report of Sharpe and Skakkebaek (1993) in which they were seeking to know whether estrogens are involved in falling sperm counts and disorders of the male reproductive tract although in the case of the present study, the

pesticides showed a type of synergism between them because they reduced more the semen volume than when they reacted alone.

On the other hand, it was also observed that, as the fish lasted longer exposed to the chemicals, the duration of sperm activity and the average life span of the sperm trail was reduced. This observation led us to question about the quality of sperm released at each very time during mating.

In general according to all the results represented in the present study, a strong mechanistic case can be made to explain how exposure of male zebrafish to the tested chemicals could disturb the quantity of sperm released as well as their motility and life span. An important question remaining to be asked is to know how should have variated the values of the F-ratio when the male fish remained for more than 2 months exposed to the chemicals. The suggested mechanism whereby exposure to the tested chemicals could induce the parameter assessed, could be offered as a hypothesis on which to focus discussion.

4.5) Ultrastructure of the testes and spermatogenesis

Semi-thin and ultra-thin sections of zebrafish (*B.rerio*) testes showed all the stages of spermatogenesis described by Manni & Rasotto (1997). The germinal epithelium of the testes of *B rerio* is organized according to the unrestricted lobular type as described by Grier (1981). Spermatocytes surrounded by Sertoli cells, form true cysts that open before the end of the spermatogenesis, leading to an asynchronous maturation of the spermatids. This type of spermatogenesis has been defined as semicystic spermatogenesis by Mattei et al (1993) as opposed to cystic in which spermatogenesis is completed within the cysts where germ cells are linked by cytoplasmic bridges.

Sertoli cells are similar to those described in several teleost species (Grier, 1993). With their veil-like lateral processes, apically joined by tight junctions, they demarcate a distinct compartment where spermatogonia and spermatocytes are housed. Their main cytoplasmic characteristic is the presence of heterophagic vacuoles, a sign of phagocytic activity for germinal cell components as also described by Grier in 1993. In the interlobular space, Leydig cells are present, showing ultrastructural features commonly found in steroid-producing cells.

In the present study, observations of semi-thin sections of zebrafish testes exposed to the different tested concentrations of DDT , Arochlor1254 and their mixture showed that the number of sperm in the lobule lumen decreased as the level of the tested chemicals increased, and as the fish remained longer exposed to them. After 2 months of exposure, in all thrice cases was the number of sperm more reduced in the lobule lumen when compared to that of the uncontaminated fish. The lipid droplets did not appear regularly but sometimes as it was the case after 1 month of exposure to 5µg/l DDT, 2 months of exposure to 0.5µg/l DDT, and 2 months of exposure to the 1:1 mixture of DDT and Arochlor 1254 tested concentrations. But this irregular presence of lipid droplets could not help to establish whether it was either an impact or not. This decrease of the number spermatids in the lumen was accompanied by the presence of numerous germ cells of the efferent duct whose interlobular space enlarged and became more pronounced. The question now is to know at which level the spermatogenesis and the cellular constitution of the lumen are disturbed by the chemicals, since according to results already obtained, the production of spermatids seemed to have been inhibited which could explain and support the results found during the sperm counts after mating events of male zebrafish. The decrease of the sperm count in lobule lumen tended to be more significant when the fish were exposed to the 1:1 mixture of DDT and Arochlor 1254. It could be per consequent proposed that DDT and Arochlor1254 could act synergically and be involved in falling the sperm count. This is a proposition which could be of great importance to the question of Givercmann & Skakkebaek in 1992 who in their report were seeking to know whether the estrogens are involved in falling the sperm counts.

On the other hand to know more about this reduction of spermatids in the lumen it is necessary to observe the ultra-thin sections of the testes in order to investigate their ultracellular constitution.

The different stages of spermatogenesis including spermatogonia were observed along the lobules of *B.rerio* testes. The epithelial cells of the testicular efferent duct system have high secretory activity. The distribution and identification of cell types within the intra and interlobular compartments of the testes of *B.rerio* revealed that within the lobules, Sertoli cells were associated with germ cells. No lipid droplets were observed regularly in the Sertoli cell cytoplasm only in some few cases as already mentioned which after the contamination was difficult to establish as abnormality. Aside from cells associated with the circulatory system of the testes, only two cell types, boundary cells and Leydig cells were observed within the interlobular tissues. These cells resided within a distinct extravascular space (plate

4d) in which boundary cells formed a discontinuous epithelial layer over the lobule surface and often did not have any contact with it in 2-dimensional electron micrographs.

During the present experiments we observed that as the fish remained longer exposed to the chemicals with increased levels, the spermatids reduced more in the lobule lumen. This reduction was accompanied by the absence almost of the phagocytosis of generating germ cells and residual bodies which are considered as one of the two functions that denote sertoli cell homology in the vertebrates, the other being the Sertoli barrier which agrees with the report of Grier in 1993.

It was also observed that the reduction of spermatids was characterized by the reduction of the terminal stage of the spermatogenesis. The round spermatids at primary stage and spermatids at second stage were the most present. The spermatids at tertiary stage were rare and it was as if the developmental stages of the spermatids were inhibited. The rarety of spermatids induced the rarety of mitochondria and cytoplasmic organelles such as rough and smooth endoplasmic reticulum and Golgi complexes after 1 and 2 months of exposure of zebrafish to 5 and 50µg/l of the tested chemicals. According to Lahnsteiner and Patzner (1990), Gardiner (1978), all these features suggest that the epithelium is involved in synthesis and secretion of proteins and sugars, and it could play a role in the process of spermatid maturation providing substances for their metabolic processes as occurs in many vertebrate species. With reference to the present results, it coul be proposed that, the rarety of the cytoplasmic organelles reduced the secretory activity of the epithelial cells, delayed the development stages of spermatogenesis and inhibited the maturation of spermatids, all leading to the production of reduced number of sperm by contaminated fish. Moreover, it is also important to mention the absence of cytoplasm heteregeneous vacuoles in the testicular duct cells which explain also the reduction of spermatids since they are probably involved in the resorption of generating spermatids, sperm, and the residual bodies cast off by developing spermatids. All these impacts described were more significant when the mixture of the pesticides were used as tested chemical, explaining only that DDT and Arochlor 1254 reacted synergically.

5) Conclusion

Laboratory studies with zebrafish provide a body of evidence which demonstrates important health effects resulting from exposure to endocrine disrupting like DDT and Arochlor 1254 at levels including current environmental levels too.

These health effects include altered development and behavior, impaired reproductive success, reduced length and altered immune system function. These effects could be the cause of cancer which may be related to in utero exposures as well , but evidence is incomplete. In many cases, the understanding of mechanisms and dose-response relationships is poor and considerable research is necessary.

However, when we weigh scientific evidence, we need to explicitly recognize that the limits of science influence both the questions we ask and how well we are able to design studies to get answers. But it has become almost possible to establish that DDT, Arochlor 1254 and their mixture are attached to a hormone receptor causing sperm count decline, and diminished reproductive success in zebrafish *(B.rerio)*. The sperm count decline could after some histological studies be due to the little number of mature sperm at the late phase of spermatogenesis. This simply explains that, the developmental stages of spermatogenesis are either retarded or do not happened at all.

The testing of the 1:1 mixture of DDT and Arochlor 1254 is found to be a reproducible method when basing on the fact that free ranging wildlife or human populations and subpopulations living in diverse environments, with complex, underlying background exposures to multiple, manmade chemicals, present study problems much more difficult to overcome. The chemical mixture also demonstrates that it was more efficient in the activity and that a synergy has occurred between DDT and Arochlor 1254 agreing with the report of Arnold in 1994 in which he stated that many chemical compounds in the environment could act synergically to bind and activate the human oestrogen recptor hER.

Biological complexities include 1) interactions among the endocrines, neurogical and immune systems, 2) interactions among genetic, environmental, and social factors- interactions which we know exist and which do not lend themselves to simplistic reductionist analysis, 3) particularly susceptible species, subpopulations, and individuals, and multiple, real world background exposures overlaid with new ones.

Some endpoints subtle, delayed like the duration of life cycle stages, or difficult to detect- and yet they may be the most important and the very things we care most about.

Our tested concentrations on DDT and Arochlor have produced a large volume of results relating to their toxicity. Even with these chemicals, there are large gaps in our understanding of mechanisms of action. Most other compounds some of which are in widespread commercial use, found virtually throughout the world, to which large populations of humans and wildlife are exposed, have been studied much less or not at all.

We have an understable interest in the mechanism of action. It is part of a long-standing scientific tradition. Mechanistic understanding satisfies our intellectual curuosity, advances research, and provides arguments for philosophical and legal debates.

Today some authors are engaged in a large global experiment. It involves widespread exposure of all species of plants and animals in diverse ecosystems to multiple manmade chemicals. And in flagrant disregard of the notion of informed consent which underlies medical experimentation and treatment, those exposed are frequently uniformed and have rarely given consent. Instead the limits of science and rigorous requirements for establishing causal proof often conspire with a peverse requirement for proving harm, rather than safety, to shape public policies which fail to ensure protection of public health and the environment.

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List of Abbreviations:

A

ANOVA	Analysis of Variance
A54	Arochlor 1254

B

BC	Boundary Cells
BCF	Bioconcentration Factor
BCF8days	Bioconcentration on the eighth day
BL	Basal lamina
B. rerio	*Brachydanio rerio*
BV	Blood vessel

C

C_f	Concentration test substance in fish
CH's	Chlorinated Hydrocarbons

CLC	Complete Life Cycle
CNS	Central Nervous System
Conc	Concentration
C_W	Concentration test substance
CWSS	Common Wadden Sea Secretariat

D

DDT	1,1,1-trichloro-2,2-bis(p-chlorophene)ethane
DIN	Deutsches Institut für Normung

E

ECD	Electron Capture Detector
Ed	Efferent duct
ELS	Early Life Stage
EPA	Environmental Protection Agency

F

F-I generation	First generation
F-II generation	Second generation

G

g	gram
GC	Gas Chromatography

H

hER	human Estrogen Receptor
hrs	hours

I

IGR	Insect Growth Regulator

L

L	Lobule lumen
LC	Life Cycle

LC50	median Lethal Concentration
LCS	Life Cycle Stage
LE	Leydig Cells
Li	Lipid droplets
LOEC	Lowest Observed Effect Concentration

M

min	Minute
ml	Millilitre
mm	Millimetre
µg/l	microgram per litre
µl	microlitre
µm	micrometre
µg/kg	microgram per kilogram

N

n	nuage

N	Nucleus
ND	Not detected
NOEC	No Observed Effect Concentration
NRCC	National Research Council of Canada

O

OC	Organochlorine
OECD	Organisation for Economic Cooperation and Development

P

PCB	Polychlorinated Biphenyl

R

RB	Residual Body

S

s	second
Sc	Spermatocyte

SD	Standard Deviation
SE	Sertoli Cell
Sg	Spermatogone
SOP	Standard Operating Procedure
Sp	Spermatid
SpI	Primary Spermatid
SpII	Spermatid at second stage of development
SpIII	Spermatid at tertiary stage of development
Subst	Substance
Sz	Spermatozoa

T

T	Time
TEM	Transmission Electronic Microscope

U

US	United States
US-EPA	United States Environmental Protection Agency

W

Wks	Weeks

APPENDICES

Appendix 1: DDT residues in the medium and fish at the dosed concentration 0.05 µg/l

Time	aquarium without fish			aquarium with fish			
		medium			medium		fish
h	ph	O_2 conc mg/l	Conc test subst(C_w) µg/l	ph	O_2 conc mg/l	Conc test subst(C_w) µg/l	Conc test subst(C_f) µg/kg
0	8.11	9.1	0.046	8.31	9.2	0.044	1.60
6	8.24	8.7	0.046	8.38	8.9	0.030	16.82
24	8.37	8.6	0.042	8.42	8.9	0.022	14.80
48	8.43	8.6	0.041	8.39	8.8	0.016	16.65
96	8.43	8.4	0.040	8.38	8.6	0.010	29.60
144	8.40	8.5	0.039	8.42	8.7	ND	26.67
192	8.42	10.9	0.037	8.42	10.8	ND	28.00

Appendix 2: DDT residues in the medium and fish at the dosed concentration 0.5µg/l.

Time	aquarium without fish			aquarium with fish			
		medium			medium		fish
h	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	Conc test subst (C_f) µg/kg
0	8.11	9.0	0.492	8.42	8.9	0.350	6.900
6	8.23	8.7	0.480	8.40	8.7	0.290	14.022
24	8.38	8.6	0.420	8.39	8.8	0.260	14.115
48	8.42	8.5	0.390	8.38	9.0	0.227	15.552
96	8.41	8.4	0.371	8.42	8.9	0.200	48.226
144	8.42	8.3	0.349	8.44	8.7	0.167	55.662
192	8.44	10.7	0.316	8.42	9.1	0.09	58.890

Appendix 3: DDT residues in the medium and fish at the dosed concentration 5µg/l

time	medium			medium		fish	
h	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	Conc test subst (Cf) µg/kg
0	8.17	8.9	4.900	7.95	8.9	3.180	15.777
6	8.23	8.5	4.820	8.05	8.6	2.910	20.150
24	8.07	8.5	4.610	8.26	8.4	2.820	190.152
48	8.27	8.4	4.022	8.34	8.5	2.415	294.038
96	8.27	8.3	3.813	8.36	8.4	1.410	360.207
144	8.23	8.6	3.714	8.37	8.6	1.041	564.580
192	8.23	10.8	3.415	8.38	10.8	0.967	727.398

Appendix 4: DDT residues in medium and fish at the dosed concentration 50µg/l

	Aquarium without fish			aquarium with fish			
time	medium			Medium			fish
h	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	Conc test subst (C_f) µg/kg
0	8.11	9.1	49.032	8.31	9.2	27.518	13.361
6	8.24	8.7	49.000	8.38	8.9	11.818	344.566
24	8.37	8.6	48.981	8.42	8.9	9.890	391.354
48	8.43	8.6	48.550	8.39	8.8	7.760	1079.106
96	8.43	8.4	48.515	8.38	8.6	6.540	2610.523
144	8.40	8.5	48.497	8.42	8.7	5.950	2686.897
192	8.42	10.9	48.420	8.42	10.9	4.200	3542.360

Appendix 5: A54 residues in medium and fish at the dosed concentration 0.05µg/l

	Aquarium without fish			aquarium with fish			
time	medium			Medium			fish
h	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	Conc test subst (C_f) µg/kg
0	8.20	9.2	0.048	8.32	9.2	0.041	2.398
6	8.37	8.9	0.047	8.35	8.9	0.035	8.489
24	8.42	8.7	0.045	8.31	8.8	0.027	11.868
48	8.41	8.6	0.041	8.39	8.7	0.018	19.757
96	8.40	8.7	0.040	8.42	8.6	0.015	20.444
144	8.42	8.9	0.039	8.41	8.7	ND	28.547
192	8.39	9.3	0.039	8.42	9.7	ND	30.590

Appendix 6: A54 residues in medium and fish at the dosed concentration 0.5µg/l

	Aquarium without fish			aquarium with fish			
time	medium			Medium			fish
h	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	ph	O_2 conc mg/l	Conc test subst (C_w) µg/l	Conc test subst (C_f) µg/kg
0	8.13	9.1	0.495	8.41	8.9	0.480	1.400
6	8.11	8.9	0.492	8.42	8.6	0.392	9.774
24	8.28	8.7	0.489	8.39	8.5	0.371	19.340
48	8.35	9.0	0.468	8.42	9.1	0.349	32.390
96	8.32	8.4	0.436	8.41	8.9	0.316	34.113
144	8.40	8.6	0.415	8.43	8.7	0.260	62.100
192	8.41	9.4	0.405	8.42	9.2	0.175	76.990

Appendix 7: A54 residues in medium and fish at the dosed concentration 5µg/l

	aquarium without fish			aquarium with fish			
time	medium			Medium			fish
h	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	Conc test subst (Cf) µg/kg
0	8.17	8.8	4.920	7.98	8.8	4.881	20.83
6	8.20	8.6	4.900	8.20	8.7	4.190	759.17
24	8.25	8.5	4.870	8.17	8.8	3.981	330.51
48	8.23	8.4	4.830	8.25	8.5	3.600	606.57
96	8.25	8.3	4.730	8.30	8.6	3.180	835.18
144	8.25	8.5	4.690	8.27	8.9	2.579	1268.66
192	8.25	8.9	4.415	8.33	9.3	2.216	1600.58

Appendix 8: A54 residues in medium and fish at the dosed concentration 50µg/l

	aquarium without fish			aquarium with fish			
time	medium			Medium			fish
h	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	ph	O2 conc mg/l	Conc test subst (Cw) µg/l	Conc test subst (Cf) µg/kg
0	8.09	9.2	49.125	8.33	9.1	28.603	21.911
6	8.13	9.1	48.730	8.35	8.9	13.201	426.760
24	8.20	8.8	48.150	8.37	8.9	11.350	498.350
48	8.37	8.5	47.802	8.36	9.2	9.735	1305.260
96	8.40	8.7	47.235	8.35	8.7	8.450	3375.271
144	8.41	10.1	46.911	8.41	8.9	7.590	3502.182
192	8.42	8.9	46.457	9.02	9.3	5.100	4005.875

Appendix 9: Average length (mm) of fish within 40 days after the development of embryos and larvae at the DDT tested concentrations.

Time(days)	Control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
2	15.52	9.40	8.11	7.75	7.33
5	18.25	12.51	9.82	8.91	7.97
10	20.72	18.50	10.52	9.91	8.20
15	26.80	19.01	10.95	9.63	-
20	25.47	21.22	11.80	10.95	-
25	26.92	20.77	14.20	11.25	-
30	27.33	22.33	17.37	12.03	-
35	27.40	24.50	19.60	15.20	-
40	28.99	24.90	22.01	19.63	-

Appendix 10: Average length (mm) of fish emerged at the different A54 tested concentrations after the embryo larval stage.

Time (days)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
2	15.52	14.57	8.19	8.07	7.64
5	18.25	18.13	9.91	9.11	8.20
10	20.72	20.65	10.75	10.68	9.95
15	26.80	26.71	12.80	11.38	10.76
20	25.47	27.02	14.31	12.25	11.35
25	26.92	26.95	14.92	12.78	-
30	27.33	27.11	17.72	13.25	-
35	27.40	27.25	19.65	16.03	-
40	28.99	28.53	22.15	19.74	-

Appendix 11: Average length of fish (mm) emerged at the different mixture of DDT and A54 tested concentrations after the embryo-larval stage.

Time (days)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
2	15.52	13.06	11.20	10.63	9.55
5	18.25	15.98	12.35	11.82	10.25
10	20.72	17.65	14.23	13.05	-
15	26.80	22.30	17.52	16.26	-
20	25.47	23.10	18.23	17.07	-
25	26.92	23.57	19.45	18.33	-
30	27.33	25.08	20.26	19.57	-
35	27.40	25.52	20.75	19.98	-
40	28.99	26.45	22.98	21.35	-

Appendix 12: Sperm count (10^3 sperm / ml) after 24 hours of exposure to the DDT tested concentrations:

Time(s)	Control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.51	12.42	11.57	10.60	5.46
40	20.31	19.74	18.77	13.94	12.30
60	21.93	21.35	21.70	15.44	14.80
120	23.67	22.95	22.67	17.2	17.71
180	24.74	24.02	23.08	20.4	19.70

Statistical test: ANOVA

F-ratio Degree of freedom (4, 20) = 0.11

Appendix 13: Sperm count (10^3 sperm / ml) after 2 weeks of exposure to the DDT tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.67	11.97	6.09	10.52	3.04
40	20.81	18.80	16.86	13.06	7.88
60	22.07	20.76	19.15	15.44	10.80
120	23.70	21.75	21.41	17.83	12.15
180	25.02	23.35	22.89	18.55	13.82

Statistical test: ANOVA

F- ratio Degree of freedom (4, 20) = 0.35

Appendix 14: Sperm count (10^3 sperm / ml) after 1 month of exposure to the DDT tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.31	12.25	5.87	5.84	1.88
40	19.98	19.53	11.74	8.85	5.15
60	22.11	21.19	13.53	9.83	7.88
120	24.10	22.75	14.29	12.09	9.48
180	25.20	23.90	14.60	13.94	10.74

Statistical test: ANOVA

F-ratio Degree of freedom (4, 20) = 0.71

Appendix 15: Sperm count (10^3 sperm / ml) after 2 months of exposure to the DDT tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.70	12.50	4.87	3.70	1.26
40	21.36	20.06	7.06	4.55	2.48
60	22.76	21.84	8.30	5.46	2.98
120	24.30	23.22	9.83	6.75	3.48
180	25.50	25.11	10.36	7.72	3.74

Statistical test: ANOVA

F- ratio Degree of freedom (4, 20) = 1.31

Appendix 16: Sperm count (10^3 sperm / ml) after 24 hours of exposure to the A54 tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.51	12.45	12.05	11.20	7.53
40	20.31	19.78	19.25	14.36	13.37
60	21.93	21.50	21.30	17.22	15.56
120	23.67	23.13	22.91	20.08	18.30
180	24.74	24.33	23.12	21.44	20.17

Statistical Test: ANOVA

F-ratio at Degree of freedom (4, 20) = 0.07

Appendix 17: Sperm count (10^3 sperm / ml) after 2 weeks of exposure to the A54 tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.67	12.11	11.23	11.06	6.34
40	20.81	19.50	18.83	15.73	8.91
60	22.07	21.25	20.76	16.75	12.73
120	23.70	22.10	21.74	18.62	14.25
180	25.02	23.52	23.12	19.39	15.03

Statistical Test: ANOVA

F-ratio at Degree of Freedom (4, 20) = 0.17

Appendix 18: Sperm count (10^3 sperm / ml) after 1 month of exposure to the A54 tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.31	12.20	11.67	10.52	5.69
40	19.98	19.56	13.36	12.91	8.38
60	22.11	21.53	15.09	14.17	10.98
120	24.10	23.37	16.21	15.03	11.65
180	25.20	24.15	17.73	15.88	11.95

Statistical test: ANOVA

F-ratio at Degree of Freedom (4, 20) = 0.33

Appendix 19: Sperm count (10^3 sperm / ml) after 2 months of exposure to the A54 tested concentrations.

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.70	12.18	9.28	6.78	3.93
40	21.36	19.63	10.33	8.53	4.50
60	22.74	21.95	11.46	9.35	5.25
120	24.30	23.72	12.19	10.61	5.74
180	25.50	25.83	12.66	11.01	6.18

Statistical test: ANOVA

F-ratio at Degree of freedom (4, 20) = 1.04

Appendix 20: Sperm count (10^3 sperm / ml) after 24 hours of exposure to the 1:1 mixture of DDT and A54 tested concentrations

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
40	12.51	9.51	7.36	6.97	3.25
20	20.31	13.25	9.45	8.37	4.36
60	21.93	16.74	11.23	10.13	4.77
120	23.67	19.34	11.89	10.78	5.10
180	24.74	21.63	12.21	10.96	5.42

Statistical test: ANOVA

F-ratio at Degree of Freedom (4, 20) = 0.98

Appendix 21: Sperm count (10^3 sperm / ml) after 2 weeks of exposure to the 1:1 mixture of DDT and A54 tested concentrations

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.67	10.2	6.80	5.76	3.14
40	20.81	13.63	9.07	7.48	4.27
60	22.07	17.01	10.98	8.97	4.60
120	23.70	19.55	11.32	9.40	4.96
180	25.02	21.75	11.60	9.89	5.15

Statistical Test: ANOVA

F-ratio at Degree of Freedom (4, 20) = 1.05

Appendix 22: Sperm count (10^3 sperm / ml) after 1 month of exposure to 1:1 mixtures of DDT and A54 tested concentrations

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.31	11.02	6.18	4.72	2.15
40	19.98	13.73	8.90	6.61	3.20
60	22.11	17.39	9.31	7.11	3.75
120	24.10	20.22	9.86	7.38	3.96
180	25.20	21.93	10.27	7.84	4.13

Statistical Test: ANOVA

F-ratio at Degree of Freedom (4, 20) = 1.58

Appendix 23: Sperm count (10^3 sperm / ml) after 2 months of exposure to the 1:1 mixture of DDT and A54 at all the tested concentrations

Time (s)	control	0.05µg/l	0.5µg/l	5µg/l	50µg/l
0	0	0	0	0	0
20	12.70	10.81	6.03	3.11	0.95
40	21.36	13.51	8.25	4.13	1.37
60	22.74	17.11	8.97	4.84	1.82
120	24.30	19.98	9.66	6.05	2.09
180	25.50	21.67	10.02	6.30	2.61

Statistical Test: ANOVA

F- ratio at Degree of Freedom (4, 20) = 2.17

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Publications:

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