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Fungicides as Endocrine Disrupters in Non-Target Organisms

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1. Introduction

In the past few decades concern has been growing about the possible consequences of environmental exposure to a group of chemicals (natural, synthetic, industrial chemicals or by-products) suspected to alter the functions of the endocrine system and consequently causing adverse health effects in an intact organism, its offspring, or (sub) population (European Commission, 2007), the Endocrine Disruptor Compounds (EDCs). Today, this concern is focused both on human health and on the impacts on wildlife and the environment, being already a priority in research and legislation within the European Union (European Commission, 1999, 2001, 2004, 2007), the US Environmental Protection Agency (Kavlock et al., 1996; U.S. EPA, 1998; Harding et al., 2006) and the World Health Organization (Damstra et al., 2002).

All vertebrate and invertebrate taxa use chemical signalling molecules (hormones). Changes of the endocrine function can occur when EDCs interfere with the synthesis, secretion, transport, action or elimination of natural hormones, which are responsible for homeostasis mechanisms, reproduction, growth and/or behaviour. These interferences can be caused by the direct binding of EDCs to hormone receptors – acting as hormone mimics (agonists) or as "anti-hormones" (antagonists) – or indirectly through modulation of endogenous hormone levels by interfering with biochemical processes associated with the production, availability, or metabolism of hormones or also by the modulation of their receptors (Rodriguez et al., 2007).

2. Endocrine active compounds and invertebrates

Although invertebrates dominate over 95% of the known animal species and represent more than 30 different phyla within the animal kingdom (Ruppert et al., 2003), potential effects of suspected EDCs on the various invertebrate endocrine systems have not been studied with comparable intensity as in vertebrates, especially in fish (e.g. Baker et al., 2009), reptiles (e.g. De Falco et al., 2007), amphibians (e.g. Kaneko et al., 2008), birds (e.g. Halldin et al., 2001), and mammals (e.g.Tabuchi et al., 2006).

Even though the issue of Endocrine Disruption (ED) in invertebrates received some scientific interest in the past, only a limited number of confirmed cases were reported (deFur

et al., 1999). These are largely dominated by investigations on insect growth regulators (IGRs), which are designed to act as EDCs for insect pest control, and by studies on the antifouling biocide tributyltin (TBT) that has been shown to induce imposex and intersex in prosobranch snails (Matthiessen & Gibbs, 1998; Sousa, 2009b). Imposex has been associated with skewed sex ratios, reduced fecundity, population declines, and local extinctions of affected gastropod populations (Gibbs & Bryan, 1986). These are perhaps the most complete examples of ED studies in wildlife populations. Further examples for ED in invertebrates are scarce and limited to laboratory studies, where effects on endocrine regulated processes in marine and freshwater invertebrates (Porte et al., 2006), and in the soil compartment have been demonstrated for some compounds (Lemos et al., 2009, 2010a, 2010b, 2010c). Endocrine changes following exposure to certain compounds may therefore be missed or simply be immeasurable at present, even though there is increasing evidence indicating that invertebrates are susceptible to ED (Porte et al., 2006).

Consequently, there is no reason to suppose that far-reaching changes as demonstrated by TBT and its effects on prosobranch populations are in any sense unique within invertebrates (Matthiessen & Gibbs, 1998).

Additionally, since many chemicals have been considered as endocrine disrupters in vertebrates and chemical signalling systems and their basic mechanisms in the animal kingdom exhibit some degree of conservatism (McLachlan, 2001) we can presume that endocrine systems in invertebrates can be subject to modulation by identical or similar compounds as in vertebrates (Pinder and Pottinger, 1998). But, and as stated before, despite their abundance and variety, relatively little is known about their endocrine systems, making data obtained by studies on endocrine disruption rather difficult to interpret.

The "Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment" report (deFur et al., 1999) summarizes 56 studies where ED may have occurred in invertebrates, although non-endocrine mechanisms are also possible scenarios for the observed effects. Effects like reduced molting frequency, reduced fecundity, elevated ecdysteroid levels, delayed reproduction, reduced size of neonates, increased brood size, mortality, molting impairment, delayed maturation, impairment of reproduction, reduced egg production, delayed brood release, reduced elimination of testosterone metabolites, retardation of regenerative limb growth, suppression of ovarian growth, differential sex ratio and super-female induction, have been reviewed in this report. This includes several studies which comprise many compounds suspected of being hormonally active on aquatic crustaceans.

With the exception of TBT, effects in molluscs, that have been associated to a locally severe impact on community levels (e.g. Blaber, 1970), and IGRs in target terrestrial insects, there are only a few field examples of ED in invertebrates. Nevertheless, much more examples for ED affecting invertebrate populations and communities can be expected.

In fact, numerous studies provide strong evidence of effects on development, fecundity and reproductive output of invertebrates that can be attributed to substances acting as EDCs (Gibbs & Bryan, 1986; Matthiessen and Gibbs, 1998; Pinder and Pottinger, 1998; Oehlmann & Schulte-Oehlmann, 2003). So, carefully targeted monitoring programs are needed because effects in invertebrates are probably widespread but undetected (Fent, 2004).

3. Pesticides as Endocrine Disruptor Compounds

In June 2000 a list of 564 potential EDCs was published in two reports of BKH Consulting Engineers, Delft, and TNO Nutrition and Food Research, Zeist, both from The Netherlands (European Commission, 2000). This list of substances was compiled having in mind the compound persistence in the environment, its production volume, the scientific evidence of endocrine disruption and wildlife and human exposure. These criteria were used to categorise the candidate substances. From these, a group of 60 compounds considered to have endocrine disrupting activity (i.e. compounds for which endocrine activity has been shown in at least one *in vivo* study) and for which a high level of concern existed with regard to exposure, deserved a special attention. These 60 compounds were included in a high priority list of EDCs proposed by the EU Commission (European Commission DG ENV, 2002). This list includes industrial chemicals such as plasticizers (e.g. benzyl-n-butylphthalate, di-n-butylphthalate, bisphenol A) or flame retardants (e.g. PBBS) but also agrochemicals or crop protection products (e.g. lindane, vinclozolin, linuron, diuron, the common metabolite of linuron and diuron, 3,4-dichloroaniline, as well as triphenyltin compounds), and biocides with antifouling properties (tributyltin compounds).

4. Fungicides as endocrine-disrupters

The increased need for pest control has made pesticide use a major issue in environmental risk assessment. Within these compounds, despite some ecotoxicology studies reporting their low toxicity to non-target species (Jansch et al., 2005; Haeba et al., 2008), many fungicides have already been reported as affecting organisms and their molecular targets and effects are now well documented.

It is not surprising that several examples of endocrine disruption have been reported for terrestrial arthropods, as several pesticides have been specially tailored to affect insects endocrine systems (IGRs), eventually co-affecting several non-target invertebrates (deFur et al., 1999). In aquatic environments the examples are scarce and reduced to the effects of TBT in molluscs that have been associated with a locally severe impact at the marine community levels (Matthiessen & Gibbs, 1998).

For this review two compounds from the EU highest priority list were selected based on their environmental occurrence as well as the existing studies confirming ED effects: the fungicides tributyltin (androgen) and vinclozolin (anti-androgen). Focusing on these compounds and their effects on non-target invertebrates, an overview of terrestrial and aquatic compartments is here addressed.

5. Case studies

5.1 Tributyltin

Tributyltin (TBT) compounds are a subgroup of the trialkyl organotin family of compounds. Of all known organotins, some of the most toxic are tributyltin compounds like tributyltin oxide (TBTO; Fig.1A) and tributyltin chloride (TBTCl; Fig.1B) (Carfi 2008). TBT compounds are organic derivatives of tin (Sn^{4+}) characterized by the presence of covalent bonds between three carbon atoms and a tin atom (Antizar-Ladislao, 2008). While inorganic forms of tin are regarded as non-toxic, these more lipid-soluble organotins can be highly toxic (Gadd, 2000).

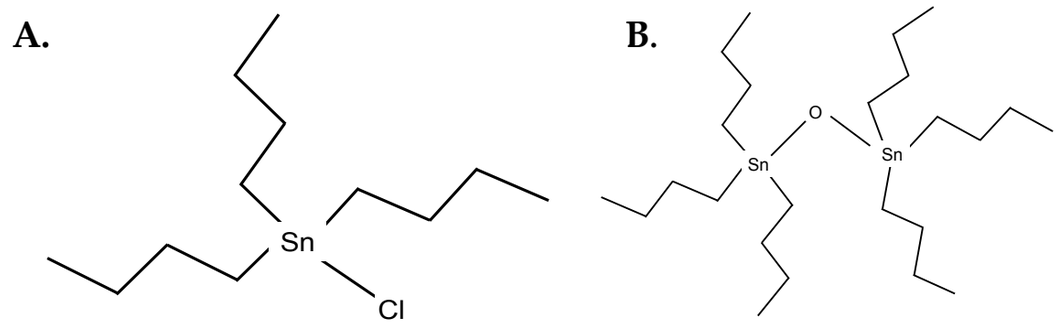

Fig. 1. Molecular structures of tributyltin chloride (A) and tributyltin oxide (B).

They are the main active ingredients in pesticides used to control a broad spectrum of organisms as they act as biocides for fungi, bacteria and insects (Mimura et al., 2008; Fent, 2006). Nevertheless, research undertaken since the early 1970s has shown that TBT is highly toxic to a larger number of non target aquatic organisms (Antizar-Ladislao, 2008). TBTs' properties were recognized in the 1950s and since then this compound has been extensively used for various industrial purposes such as slime control in paper, as a wood preservative, as a polyvinyl chloride (PVC) stabilizer, and as fungicide in agriculture (Mimura et al., 2008). In the 1970s, TBT paints widely replaced copper-based paints due to their superior performance in terms of efficacy and duration (Sonak, 2009). Since then, TBT has been used mostly as an antifouling agent in marine paint formulations to prevent the attachment of barnacles and slime on boat hulls and aquaculture nets (Kannan et al., 1998).

Due to its widespread use as an antifouling agent in boat paints, TBT is a common contaminant of marine and freshwater ecosystems. Its damaging consequences to marine ecosystems were recognized in early 1980s as the cause for the decline of some marine molluscs (Smith, 1981; Waldock & Thain, 1983; Bryan et al., 1986). As a result of field evidences of negative ecological impact of organotins, the European Union published a Directive (89/677/CEE) banning TBT application on ships smaller than 25 m. On the assumption that TBT concentrations in the open sea were too low to cause effects, there were not many restrictions on the use of organotins in larger ships. However, a similar impact in the open sea has been shown for TBT with incidence being correlated with shipping density (Santos et al., 2002). Thus, the International Maritime Organization banned the application of TBT-based paints in 2003 and called for a global agreement for total prohibition of the presence of organotins on ship hulls in 2008 (International Maritime Organization, 2001).

TBT from hulls and nets can be adsorbed onto suspended particles in the water, sediment and biota (Gadd, 2000). Subsequently it is readily incorporated into the tissues of filter-feeding zooplankton, invertebrates and eventually higher organisms such as fish and mammals where it accumulates (Antizar-Ladislao, 2008). Despite the present restrictions, TBT and its degradation products will not disappear immediately from the marine environment, and it can be expected that TBT will remain in waters and sediments for long periods of time because of the moderate to high persistency in anoxic sediments and its' widespread presence, as was confirmed by the work of Sousa and co-workers along the Portuguese coast (Sousa et al., 2009). Together with its lipophilicity, it tends to accumulate in oysters, mussels, crustaceans, molluscs, fish, and algae favouring the bioconcentration up the marine predators' food chain (Santos et al., 2009; Cruz et al., 2010).

Because of all the above mentioned, recently organotins have been considered as the most toxic substance ever introduced into the marine environment so far (Fent, 2006; Guo et al., 2010; Antizar-Ladislao, 2008; Sonak, 2009), and due to their use in a variety of industrial processes, their environmental fate and ecotoxicity as well as human exposure are topics of current concern.

In a study conducted by Guo et al (2010) on western clawed frog embryos (*Xenopus tropicalis*) the authors suggested that TBT might be the cause of several malformations. These include the loss of eye pigmentation, enlarged trunks and bent tails, in the presence of 50 ng/L of TBTCl after 24 hours of exposure. This is particularly relevant since the concentrations of TBT in open water, bays, estuaries, lakes and freshwater harbors commonly exceed this concentration with the highest TBT values found near marinas and seaports (Fent, 2006). At higher concentrations TBT may be lethal to several marine and freshwater species. Short and Thrower (1987) reported that the 96 hour LC50 for juvenile Chinook salmon (*Oncorhynchus tshawytscha*) is 1.5 µg/L.

TBT is known as an endocrine disruptor promoting adverse effects in organisms on diverse levels of biological organization (Guo et al., 2010). Matthiessen & Gibbs (1998) reported an interference with hormone metabolism, increasing the androgen levels of snails exposed to TBT. One of the best-documented and iconic adverse impacts of TBT in non-target organisms is imposex in molluscs. This pathology occurs when male sex characteristics and organs, such as penis, *vas deferens*, and seminiferous tubules are superimposed on normal female resulting in female sterilization, and even with spermatogenesis occurrence. Such pathology consequently has obvious impacts population dynamics (Gibbs et al. 1991). The first evidences linking TBT to imposex were reported in 1970 for the dog-whelk, *Nucella lapillus*, in the UK (Blaber, 1970). Since then, several studies have related TBT to the worldwide decline of marine mollusks (e.g. *Nassarius*, *Ilyanassa*, *Ocenebra* and *Urosalpinx)* in costal areas due to imposex (Gibbs et al., 1991). Administrating testosterone to the snail *Euchadra peliomphala* resulted in a stimulation of the development of male sex characters in female and castrated male gastropods (Takeda, 1980; Spooner et al., 1991). These authors also report increased testosterone titres in *N. lapillus* exposed to this fungicide. These findings have led to the hypothesis that the increased levels of testosterone in TBT-exposed organisms are responsible for the imposex development. The precise mechanism by which increased levels of testosterone are produced has not been fully described, but the weight of evidence suggests that TBT acts as a competitive inhibitor of cytochrome P450-mediated aromatase (Bettin et al., 1996). In laboratory tests, imposex was also reported from concentrations below 1 ng of TBT/L in the mud snail, *Ilyanassa obsoleta* (*Gooding* et al., 2003), and the dog-whelk, *N. lapillus* (Gibbs et al., 1988). There are still uncertainties regarding the sensitivity of the endocrine function in different genders and developmental stages in invertebrates exposed to EDCs (Rodriguez et al., 2007). Nevertheless, some authors agree that the hormonal impacts of TBT and EDCs in general may differ according to the specific life stage at which exposure occurs (e.g., embryolarval stages, gonadal development, etc.) (deFur et al., 1999; Lemos et al., 2009). Generally, the larvae/neonates of any tested species are more hormonally sensitive to tributyltin exposure than are the adults. For example, the adult female dog-whelk, *N. lapillus*, revealed imposex signs at concentrations as low as 5 ng of TBT/L while young and sexually immature females were more sensitive than adults with concentrations of 1 ng of TBT/L inducing the growth of penis and *vas deferens* tissue (Gibbs et al., 1987).

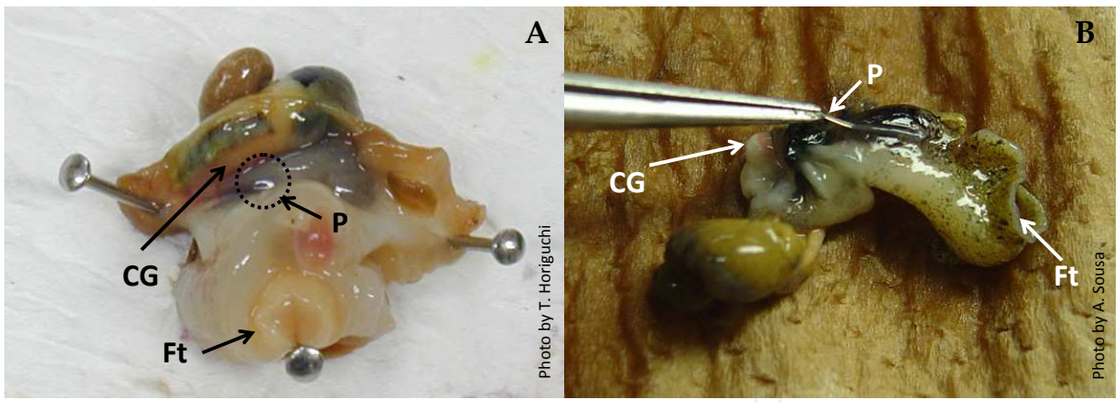

Fig. 2. Female gastropods exhibiting imposex: *Nucella lapillus* (A), and *Nassarius reticulatus* (B). CG - capsule gland; Ft - foot; P – penis. Photographs are courtesy of Prof. T. Horiguchi and Dr. A. Sousa.

Deviations from normal limb regeneration in the prawn, *Caridina rajadhari* (Reddy et al. 1991), and in the fiddler crab *Uca pugilator* (Weis et al., 1987), have been observed in laboratory experiments involving TBT exposure. Nevertheless, despite the findings and the known hormonal regulation of the molting process, it has been argued whether these effects are due to endocrine disruption rather than regular systemic toxicity. Unless specific parameters such as hormone levels are assessed no accurate conclusion can be drawn (Barata et al., 2004; Lemos et al 2009).

Levels in harbour and port waters prior to restrictions on TBT use in antifouling paints have shown levels higher than 500 ng/L in North American and European marinas. For example, one year before the UK ban (1986), TBT concentrations in Wroxham Broad and at the nearby River Bure boatyard were 898 ng/L and 1540 ng/L, respectively (Waite et al., 1989). They were significantly higher than in open surface waters, bays and estuaries where commonly values of up to 50 ng/L were observed (Fent, 2006). Albeit this regulation for the use of TBT and consequent general decrease in environmental TBT levels (Antizar-Ladislao, 2008), recent surveys still account for levels higher than those reported to elicit effects at a global scale: for instance, 32 ng TBT/L in South Korea (Sidharthan et al., 2002), 2–160 ng/L in Japanese coastal waters (Takeuchi et al., 2004), in the UK 10–78 ng/L were detected in marinas and harbors in 1998 (Thomas, 2001), and 200 ng/L in ferry ports in Corsica, Italy (Michel et al., 2001).

The impact of TBT in freshwater systems has been studied at a much lesser extent compared to estuarine and coastal environments. As still, endocrine effects have also been shown for freshwater molluscs. For example, after a 3 month exposure to a concentration of 50 ng of TBT/L, the giant ramshorn snail, *Marisa cornuarietis*, showed recognizable morphological characteristics of imposex development (Shulte-Oehlmann et al., 1995). Despite the scarce information (possibly reflecting a reduced concern about the impacts in this ecossystem), Schulte-Oehlmann (1997) reported concentrations in European lakes up to 930 ng of TBT /L in water and reaching 340 µg of TBT/g wet weight in sediments of River Elbe in Central Europe.

For the soil compartment, to our knowledge, there are no reported data on the effects of TBT to edaphic organisms.

5.2 Vinclozolin

Vinclozolin [Vz, 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione; Fig.3] is a non-systemic dicarboxymide fungicide, manufactured by BASF and commercially sold under the names Ronilan®, Curalan®, and Ornilan®. It is efficient in controlling plants and fruit diseases caused by *Botrytis* spp., *Monilia* spp., and *Sclerotinia* spp. (Bursztyka et al., 2008) that affect crops such as lettuce, raspberries, beans and onions (Price et al., 2007). This fungicide is widely used in the United States of America and throughout Europe. In Britain, as well as in Germany, up to 50 tonnes of Vz are used each year and it was estimated that in 2002, in the USA, 2,330,738 US dollars were spent on this compound for crop protection (Gianessi & Reigner, 2005).

When sprayed as Ronilan®, at the maximum recommended application rate, the concentration of Vz in the soil is 1 mg active ingredient (a.i.)/kg (assuming that 70 % of the fungicide will reach the surface and is homogeneously distributed over the top 5 cm soil layer and the soil bulk density is 1.4 kg/dm^3) (Lemos et al., 2009). Vinclozolin has a low to moderate persistence in soil, with reported half-lives from 28-43 days in the laboratory up to 34-94 days in the field and 6-12% of the original compound is present after 1 year (U.S. EPA, 1991; IUPAC, 2006). Despite this low-persistency of Vz, a reported increase of mortality with exposure time (Lemos et al., 2009) may be due to the increased concentration of its' three major metabolites (out from 15): 2-[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1), 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), and 3,5-dichloroaniline (DCA), which are reported to be more active than the parent compound (Kelce et al., 1997; Anway et al., 2005; Kavlock and Cummings, 2005) and have half-lives ranging from 179 to >1000 days (U.S. EPA, 2000). It has been reported that these metabolites may be produced both spontaneously in the presence of aqueous buffers and by biotransformation of Vz (Bursztyka et al., 2008). M1 and M2 are able to bind to the androgen receptor (Vinggaard et al., 1999) and competitively inhibit the binding of androgens to the human androgen receptor (Kelce et al., 1997; Kavlock and Cummings, 2005). Concerning the parent compound, it is known that it inhibits vertebrate testosterone-induced growth of androgen-dependent tissues (Kang et al., 2004).

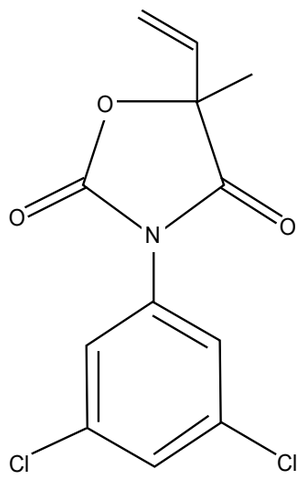

Fig. 3. Molecular structure of vinclozolin.

On plant leaves, Vz is detectable as the parent compound but does not wash off, since it is more soluble in oil than in water. This implies that Vz residues are commonly found on food (Szeto et al., 1989). Metabolites have also been found in human food (Gonzalez-Rodriguez et al., 2008).

Vinclozolin endocrine disruptor effects include induction of Leydig cell tumors, reduction of ejaculated sperm counts and prostate weight, and delayed puberty in exposed rats. One major concern is that Vz causes transgenerational effects. F1 to F4 generations of male rats exposed to Vz at the time of gonadal sex determination developed prostate disease, kidney disease, immune system abnormalities, spermatogenesis abnormalities, breast tumour development, and blood abnormalities as hypercholesterolemia, which have been associated with an alteration in the epigenetic programming of the male germ line (Anway et al., 2006; Anway & Skinner, 2008). Similar effects have been shown for pregnant rat exposed to Vz, where a transgenerational increase in pregnancy abnormalities and female adult onset disease states are promoted (Nilsson et al., 2008).

The existing information supports the hypothesis that Vz steroid-mediated actions in vertebrates have similar sub-lethal effects in invertebrates. In *Daphnia magna* it induces a decrease in the number of newborn males (Haeba et al., 2008). In molluscs Vz was shown to cause female virilisation (imposex development) and reduction of accessory sex organ expression in the fresh water snail *Marisa cornuarietis* and two marine prosobranchs *N. lapillus* and *Nassarius reticulatus* (Tillmann et al., 2001). Snails were exposed to nominal concentrations of Vz ranging from 0.03 to 1 µg/L for up to 5 months. In exposed juvenile *Marisa*, males had a slight decrease in the male accessory sex organ, particularly the penis and penis sheath. This response was only detected during the first 2-3 months of exposure for the lowest concentrations (0.03 and 0.1 µg of Vz/L), and was reversible once they attained puberty. Adult male *N. lapillus* exposed to the fungicide developed shorter penis, smaller prostate gland, and there were less males with ripe sperm stored in the seminal vesicle. Compared to the reported effects of estrogens and androgens on these two same species, these anti-androgenic responses seem to be less drastic, and might not have any biological effect at the population level (Tillmann et al., 2001). The immobilising effect (EC50, 48h) of Vz on the American oyster (*Crassostrea virginica*) and *D. magna* was reported to be 3.2 mg/L and 3.65 mg/L, respectively. For the opossum shrimp (*American bahia*) the LC50 (96h) was 1.5 -2.1 mg Vz/L (U.S. EPA, 2000).

Studies of Vz effects in the soil compartment are scarce. Vinclozolin has been reported as non-toxic to earthworms (Tomlin, 2003), but the most complete and extensive research was performed in a terrestrial isopod (Lemos et al. 2009, 2010a, 2010b, 2010c). In *Porcellio scaber*, Vz exhibited ecdysteroidal activity in ecdysone endogenous levels in a concentration-dependent way (Lemos et al., 2009). The results from this study demonstrated that the fungicide caused endocrine disruption in the isopod with an ecdysteroid up-regulation resulting in molting disturbances being further related to developmental and reproductive toxicity which enabled to suggest a causal link to ED in this class of organisms.

Since a sharp rise of ecdysteroid followed by a decrease of the hormone level triggers the ecdysis process (Bodar et al., 1990), when basal concentration of 20-hydroxyecdysone (20E) is maintained at high levels (hyperecdysonism) the shedding of the old cuticle is impaired and mortality due to incomplete ecdysis occurs (Fig. 4). Therefore, molting behaviour was associated with hyperecdysonism, delaying molt and in many cases impairing the molting process, and death surmounting at higher toxicant concentrations (1 g of a.i. Vz/kg of soil) (Lemos et al., 2009).

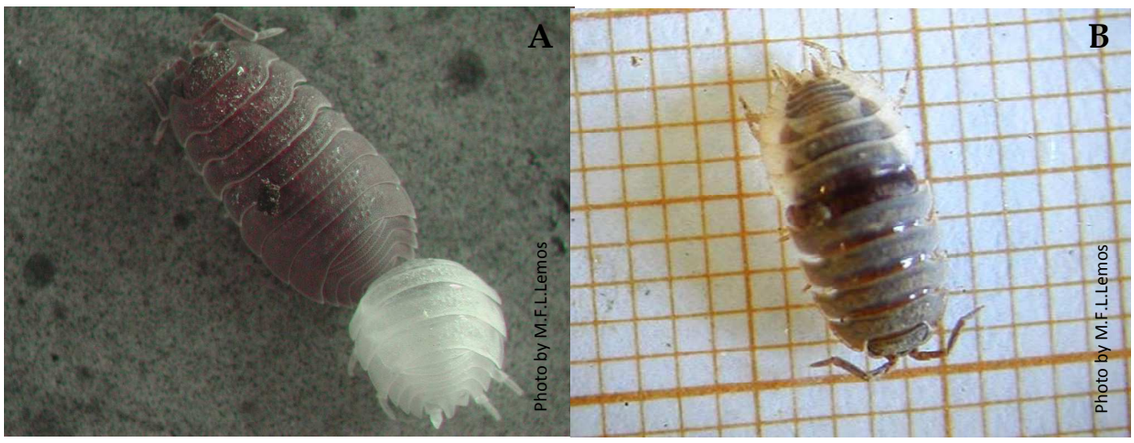

Fig. 4. Isopod *Porcellio scaber* and shedded cuticle after posterior half molt (A), and dead animal within the unshedded old cuticle after failed posterior half molt, after exposure to vinclozolin contaminated soil (B).

The same authors (Lemos et al. 2010a) also reported that young isopods respond with reduced growth at lower concentrations (LOEC of 100 mg a.i. Vz/kg soil) compared to adults (LOECs 300 mg a.i. Vz/kg soil). The increased sensitivity of the juvenile life stage may be either due to easier absorption of the toxicant through their relatively larger body surface/volume ratio and thin cuticle, or due to their lower capacity to metabolize the contaminants (Fischer et al., 1997; Lemos et al., 20010a) but another possibility pointed was the differential life-stage endocrinology.

Vinclozolin also elicited overall reproductive toxicity to *P. scaber* (Lemos et al., 2010b) decreasing the reproductive allocation for exposed females, the number of pregnancies, and the number of juveniles, while increasing the percentage of abortions. It induced a decrease of the brood period, with the isopods releasing juveniles almost 43 hours earlier at 100 mg a.i./kg dry soil and five days earlier at 300 mg a.i./kg dry soil. These two factors together considerably reduced the total juvenile output.

One of the reasons attributed to this impairment was the increased 20E titres (Lemos et al., 2009) that have been previously correlated with increased vitellogenin - a key protein of extreme importance in crustacean reproduction - synthesis and uptake in developing oocytes (Gohar &d Souty, 1984).

The molecular effect of Vz was assessed by differential protein expression of Vz exposed *P. scaber* in the gut, hepatopancreas, and gonads (Lemos et al., 2010c). In this study it was possible to detected up-regulated proteins at concentrations as low as 10 mg a.i./kg soil in the testes while for hepatopancreas this was only possible at concentrations equal to 1000 mg a.i./kg soil. Proteins from the heat shock protein family, Hsp70 (known as ubiquitous stress response proteins, anti-apoptotic, and protects cells from cytotoxicity and inhibiting cell death induced by several agents) were over-expressed at the lower concentration of Vz in the testes of organisms exposed to Vz (around 160% increase after exposure to 10 mg a.i./kg and around 130% at 30 mg a.i./kg).

Vinclozolin up-regulated arginine kinase (around 150% at 1000 mg a.i./kg for Vz) in the isopods' hepatopancreas. This enzyme is involved in the cellular energy metabolism,

suggesting an increase of resources allocated to the activation of metabolic processes related to detoxification and the metabolisation of energy reserves to provide for these processes (Lemos et al., 2010c).

The fact that the gonads showed increased protein expression at concentrations higher than 10 mg a.i./kg soil (Lemos et al., 2010c), suggests that testes are more susceptible to these compounds than other organs. Thus, male isopod reproductive traits may therefore be especially susceptible and sensitive to this fungicide. Previous studies have also stressed the gonads and reproductive traits as preferential targets by EDCs in vertebrates (Navas and Segner, 2006).

6. Conclusions

Although TBT levels have been decreasing in the last decades, mainly due to restrictions in its use, it is still present at ng/L levels in the environment (Fent, 2006). Additionally, marine prosobranch gastropods and other invertebrates are extremely sensitive to TBT contamination, and imposex can be elicited in some species at concentrations of < 1 ng TBT/L (Gibbs et al. 1988). Moreover, TBT is very persistent in sediments being is considered as the most toxic substance ever introduced into the marine environment and a major threat to the environment for many years (e.g. Fent, 2006) since its sediment concentrations (up to several mg/kg sediment) are still highly toxic to benthic fauna while organotins resuspension is possible through storms or dredging leading to an increase of organotin levels in the water column (Unger et al., 1988). Additionally one should not forget that organotins residues such as TBT can also be transferred to humans via dietary uptake. Sousa and co-workers predict that in traditional seafood-consuming countries the estimated Sn daily intake is high enough to cause damage to humans (Sousa et al., 2009b), making TBT contamination more than "just" an environmental issue of concern.

Vinclozolin is classified as very toxic (EC50 < 1 mg/L), and it has been shown to have adverse effects in the laboratory on aquatic snail at concentrations below 1 µg/L. Nevertheless, Vz is non-persistent in the environment, and degrades rapidly, particularly under alkaline conditions (Tomlin, 2003; Ueoka *et al*., 1997). Moreover, a survey of German ground and surface waters found only 1% of water samples to have detectable levels (>0.1 µg/L) of Vz (Funari *et al*., 1995). Mediterranean estuarine waters have also been investigated for fungicide contamination, and from the sites examined only the River Po in Italy had detectable levels of vinclozolin (Readman *et al*., 1997).

In the soil compartment, despite these reported severe effects in isopods, the concentrations that elicit effects are far from ecological relevant concentrations.

So far there is no clear evidence suggesting effects of Vz on the terrestrial or aquatic ecosystems. Nevertheless, in Europe, the use of this substance is no longer authorized according to EC Directive 91/414.

Despite the examples showing that invertebrates are susceptible to ED, endocrine changes following exposure to certain compounds may be missed due to scarce knowledge of invertebrate endocrinology or due to very low concentrations of certain compounds (below detection limits) eliciting ED effects. In fact there is no reason to suppose that the array of endocrine changes such as the ones demonstrated for TBT and Vz are in any sense unique

and most certainly similar effects are plausible and to be expected in most invertebrate species.

Through small biochemical and molecular changes, these contaminants may however interfere with different systems (e.g., reproductive, endocrine, immunological, and nervous) in different life stages of non-target species, causing medium and long term effects at the population level. Due to the nature of the ED mode of action, the consequences of the exposure to such class of compounds in the communities' structure and function are thus not always immediate and can be extremely hard to predict.

As said before many fungicides can behave like EDC, and because ED effects at the population and community levels might be only detected after several generations exposed to sub-lethal levels of pollutants, there is a need to develop and validate quantifiable tools for the identification of ED effects of fungicides and pesticides in general. It is our conviction that this has to be achieved with a mechanistic approach where alterations of hormonal vital processes in particular species are assessed. For that it is vital to conduct long term chronic assays where biochemical, organismal and reproductive parameters are measured and compared with changes in hormone levels of invertebrate species. It is not an easy task but due to the expected increase in fungicide production and utilization in the next decades, it is critical to adopt this kind of integrative approaches to better understand the mechanisms that link endocrine level responses to population- and community-level processes and to improve environmental risk assessment of these compounds

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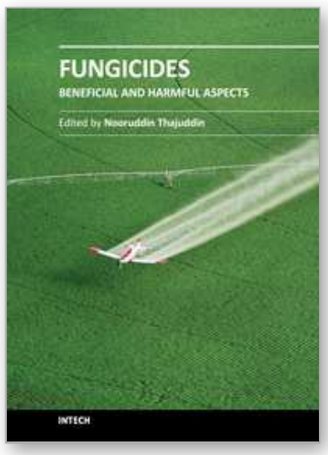

Fungicides - Beneficial and Harmful Aspects
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Fungicides are a class of pesticides used for killing or inhibiting the growth of fungus. They are extensively used in pharmaceutical industry, agriculture, in protection of seed during storage and in preventing the growth of fungi that produce toxins. Hence, fungicides production is constantly increasing as a result of their great importance to agriculture. Some fungicides affect humans and beneficial microorganisms including insects, birds and fish thus public concern about their effects is increasing day by day. In order to enrich the knowledge on beneficial and adverse effects of fungicides this book encompasses various aspects of the fungicides including fungicide resistance, mode of action, management fungal pathogens and defense mechanisms, ill effects of fungicides interfering the endocrine system, combined application of various fungicides and the need of GRAS (generally recognized as safe) fungicides. This volume will be useful source of information on fungicides for post graduate students, researchers, agriculturists, environmentalists and decision makers.

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