Bioaccumulation of thiram in *Mytilus galloprovincialis* and its effect on different tissues

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**Summary** The accumulation of thiram, a characteristic dithiocarbamate fungicide, in *Mytilus galloprovincialis* soft tissues and its effect on bivalvian DNA integrity has been examined through a series of *in vivo* exposures of mussels to 0.1, 1.0 and 10.0 mg thiram/L in saltwater for 48 h. Regarding bioaccumulation of the fungicide, a dose-dependent increase of the sodium adduct ion of thiram was observed in mussel soft tissues after the end of the exposures. This identification/quantification of thiram and one of its metabolites was achieved by Liquid Chromatography Mass Spectrometry (LC-MS) analysis. Regarding DNA integrity (DNA Single Strand Breaks) these were strongly dose-dependent. They were also tissue-dependent in concordance with the different susceptibility of certain tissues to pollutants and the levels of metabolism that takes place in them. Further investigation in oxidative and apoptotic DNA damage revealed that a) oxidative stress was evident in all the tissues examined b) apoptotic cell morphology was detected in gill and digestive gland. Imbalance of the antioxidant/prooxidant status in favour of the latter and initiation of apoptosis may be a causative mechanism of DNA damage in *M. galloprovincialis*, as it has already been shown for DNA damage caused by thiram in mammals.

*Additional Keywords:* apoptosis, dithiocarbamates, LC-MS, mussel, oxidative stress, Single Strand Breaks

**Introduction**

The dithiocarbamate class (DTCs) of fungicides comprises a group of plant protection products (PPP) which are widely used worldwide. They are the main group of fungicides used to control approximately 400 pathogens of more than 70 crops and are registered in all the EU member states and many other countries (18). They are of relatively low acute toxicity for humans with an average LD50 of more than 2523 mg/kg bw (41). Thiram (tetramethylthiuram disulphide) is one of the most characteristic dithiocarbamates. It was synthesized in 1931 and since then has been extensively used as a fungicide (41), as a seed treatment in maize, cotton and cereals (25, 35), as an accelerator and vulcanizing agent during rubber processes as well as for treatment of human scabies and in antiseptic soaps and preparations (21, 22). It is also an intermediate metabolite of two other dithiocarbamates—ferbam and ziram (41).

Several analytical methods are available for the detection and quantification of thiram and its metabolites. Gas chromatographic techniques have been applied with determination of carbon disulphide (CS₂), the product of the rapid degradation of DTCs. However, these methods are laborious and this has directed scientists to the exploitation of LC-MS methods which offer the advantage of the direct determination of DTCs and thiram in particular.

Thiram, like the majority of dithiocarbamates, exerts its toxic action via creation of metabolites of carbon disulphide (43) and it is of low mammalian toxicity (17). However, in certain animal models it has caused hepatotoxicity (24, 25) and adverse developmental and reproductive effects (24, 27, 40). It has also caused eczema, contact dermatitis and skin lesions to exposed workers (21, 22, 40).

Since thiram is so extensively used, it is commonly found in aquatic environments (31). According to U.S. Environmental Pro-
tection Agency “thiram is expected to be sufficiently mobile and persistent in some cases to reach surface waters in concentrations high enough to impact aquatic life” (20). Moreover, it may not be readily catabolised and it may persist in soil for several weeks.

A series of in vivo experiments have shown that thiram may affect certain biological or biochemical aspects in aquatic organisms namely cladocerans (31), fish (6, 42) and mussels (15). The aim of the present project was to test for bioaccumulation and effects of thiram, which is in vivo non-clastogenic for mammals (19) in the model marine mussel of the Mediterranean Sea Mytilus galloprovincialis, in relation to tissue and dose. Following positive results, mechanistic aspects of this interaction have been sought (oxidative DNA damage and early apoptotic DNA damage). These pathways are implicated in thiram toxicity in mammals but they have not yet been examined in bivalve species.

Materials and Methods

Test animals
Commercially available Mytilus galloprovincialis from a mussel farm in Evia, Greece were purchased. Mussels of similar size (8-9 cm) were kept in continuously aerated glass aquaria of 15 L saltwater (salinity 33‰) at ambient temperature of 25°C, under natural light. The mussels were fed with approximately 0.05 g powdered Spirulina (M.Rohrer, Netherlands) every day. Nitrate and nitrite levels were periodically checked (API pharmaceuticals, USA) and they did not exceed 0.5 and 0.25 μg mL⁻¹ respectively. The molluscs were acclimatized in laboratory conditions for at least 5 days before the beginning of the experiments. These species absorb quickly contaminants from their environment but they depurate in an equally rapid manner when found in clean water (2).

Fungicide and Solvents
A stock solution of 10 mg thiram/L was prepared fresh in distilled water from commercially available thiram 80% w/w and was diluted to nominal concentrations of 0.1, 1.0 and 10.0 mg thiram/L in saltwater. Thiram analytical standard was purchased from Fluka (Buchs, Switzerland) and a stock solution of 100 μg mL⁻¹ was prepared in methanol. The working solutions were prepared from this stock solution in methanol. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany) and were LC-MS grade. Ethyl acetate (pro analysi) was also purchased from Merck (Darmstadt, Germany).

Experimental set up
Healthy mussels from the aquaria were exposed in 2 L glass beakers (Simax, Czech Republic) at a density of 4 mussels/beaker to final concentrations of the agrochemical stated in 2.2. The exposures lasted for 48 h after which the animals were sacrificed. The water was changed every 12 h and was spiked with thiram after each renewal. During the exposure the animals were not fed. The general condition of the animals and the mortalities were recorded daily.

Chemical analysis

Soxhlet Extraction
Lyophilized mussels (7 g) were placed with equal amount of sodium sulfate at the bottom of the Soxhlet apparatus covered with glass wool. The solvent of choice was a mixture of ethyl acetate/hexane (1:1 respectively, 200 mL) and the extraction time was 4 h. After the extraction the solvent was removed in vacuum and the mixture was reconstituted with ethyl acetate (2 mL).

Removal of sulfur compounds and Solid Phase Extraction (SPE)
The removal procedure was the following: 1 g of activated copper was added to the reconstituted mixture (2 mL) and stirred vigorously for 2 min. Centrifugation (Heraeus Labofuge 400R Thermo Electron Corporation) of the mixture was followed by the separation of the organic phase. Then the organic phase was passed from Flori-
sil SPE cartridge (Waters, SEP-PAK® Cartridges), filtered (Whatman, Puradisc™ 25 TF filters, 0.45 μm), evaporated under a gentle stream of nitrogen, reconstituted with acetonitrile (0.5 mL) and 10 μL were injected to Liquid Chromatograph Mass Spectrometer (LC/MS).

**Experimental Procedure for Extraction of Thiram from Water Samples**

An aliquot of 2 mL of water sample was mixed with 2 mL of ethyl acetate (vortex, a MS1 Minishaker, IKA) for 2 minutes. Then the mixture was placed in an appropriate falcon tube (15 mL) and centrifuged at 4000 rpm for 5 min, at 4°C. The organic layer was collected, filtered (Whatman, Puradisc™ 25 TF filters, 0.45 μm), evaporated under a gentle stream of nitrogen, reconstituted with acetonitrile (0.5 mL) and then an amount of 10 μL was injected for analysis to the Liquid Chromatograph Mass Spectrometer.

**Liquid Chromatography Mass Spectrometry**

A Shimadzu (Kyoto, Japan) LCMS-2010 EV Liquid Chromatograph Mass Spectrometer instrument was used with the LCMS solution version 3.0 software consisting of a SIL-20A prominence autosampler and an SPD-M20A diode array detector. The latter were coupled in series with a mass selective detector equipped with an atmospheric pressure ionization. The LC separation was achieved working in positive Electron Spray Ionization (ESI) mode, on a Shim-Pack XR-ODS 2.2 μm, 100×4.6 mm i.d. chromatographic column using a gradient system consisting of methanol and water. The flow rate was set at 0.8 mL min⁻¹ and the column gradient program consisted of 40% methanol and 60% water, ramped linearly over the course of 7.5 min at 70 % methanol. Then methanol returned in the course of 2 min at 60% concentration and the mobile phase was held at that composition from 9.5 min to 15 min.

**Validation procedure**

For the validation procedure the following parameters were determined: linearity, repeatability, reproducibility, analytical Limit of Detection (LOD) and Limit of Quantification (LOQ), recoveries and matrix dependent variations as it is established by the EU guidelines. Linearity and matrix effect were assessed by analyzing standard solutions and matrix matched standards at six points in the range of 0.20-7.88 μg mL⁻¹ to cover the expected range of concentrations in samples. Recovery values derived from fortified experiments at two levels of concentration and were considered as the measure of the trueness of the analytical method. For repeatability and reproducibility studies of the LC-MS methodology, five replicate determinations on the same day and on five different days of a standard solution (0.78 μg mL⁻¹ of thiram) were performed. Repeatability and reproducibility is considered acceptable when relative standard deviation values (RSD%) are < 20%. Based on the statistical definition $(S/\alpha)\sqrt{x}$ and $(10S/\alpha)\sqrt{x}$ of the LOD and LOQ respectively, they were determined at the concentration levels ranging from 0.20 to 7.88 μg mL⁻¹. $S/\alpha$ represents the residual standard deviation and $\alpha$ is the slope of the respective calibration plot.

**Tissue preparation for biochemical assays**

The three main tissues of mussels (gill, digestive gland and haemolymph) were extracted from sacrificed animals after the end of the experiment. Briefly, for gills, the valves of the mussel were fully opened with a metallic scalpel and wet gill tissue (< 0.2 g) was extracted. The gill was gently disaggregated in HEPES buffered saline (2 mL). After centrifugation a fraction of the pellet was rediluted in saline (100 μL) and kept on ice until further processing. For haemolymph the valves of the mussel were slightly opened and haemolymph was abstracted from the posterior adductor muscle according to Rank and Jensen (36) with a 23G needle and a 1 mL syringe (PiC Insumed, Italy). Approximately 100 μL were abstracted from each animal and used without further treatment. Digestive gland suspension preparation was performed according to Birmelin et al. (8) with minor modifications. Briefly,
digestive glands (< 0.2 g) were abstracted from the animal and cut in small pieces with surgical scissors. The pieces were gently dis-aggregated in HEPES-NaOH buffered saline (10 mL) and agitated on a rocking platform (Innova2000, Brunswick Scientific) on ice for 2 h. Every 30 min aliquots of cell suspension (2 mL) were taken out and replaced with new HEPES-NaOH buffered saline. All the aliquots from the same animal were finally pooled (8 mL). After centrifugation a fraction of the pellet was rediluted in saline (100 μL) and kept on ice until further processing. The preparations provided whole and single cells with negligible additional damage.

**Single Cell Gel Electrophoresis on mussel tissue (Comet assay)**

A fraction of the cell suspension (15 μl) was mixed with 150 μL low melting point agarose. The mixture was spread on an agarose-precoated slide and lowered in lysis buffer (NaCl 2.5M, Na₂EDTA 0.1M, Tris base 10 mM, Triton-X 1% v/v and DMSO 10% v/v, pH 10.0) for 1 h at 4º C in the dark. Two slides per animal were prepared. The slides were then rinsed in distilled water (1mL/slide) and left in electrophoresis buffer (pH 13.0) in a horizontal electrophoresis tank (Cleaver Scientific, Ltd) for 30 min. The slides were subsequently subjected to electrophoresis at 25 V for 20 min, neutralised with Tris buffer (Tris 0.4M, pH 7.5) and stained with propidium iodide (2.5 μg/mL) (Research Organics, Cleveland, USA). Each slide was analyzed using a fluorescent microscope (Zeiss AxioCam MRC, Carl Zeiss Inc., Germany) at 200 x magnification, with an excitation filter of 515-560 nm and a barrier filter of 590 nm and scored using an image analysis package (TriTekCometScore©). 40 randomly selected nucleoids were analyzed per slide in two slides so that a total of 80 cells (per animal) were scored.

**Modified Single Cell Gel Electrophoresis (Comet coupled with formamidopyrime-dine glycosylase)**

The procedure was as described in 2.6 with the exception that, after lysis and before unwinding in high pH buffer, the slides were rinsed 3 times in 1mL of Fpg buffer (KCl 0.1M, HEPES 40 mM, Na₂EDTA 0.5mM, bovine serum albumin 0.2 mg/mL, pH 8.0) each. Four slides per animal were prepared. One slide per pair was incubated with one unit of Fpg enzyme (AMS Biotechnology, UK) in Fpg buffer (50 μL) for 1 h as described by Collins et al. (14). The remaining slide of each pair was incubated with 50 μL Fpg buffer only. 80 randomly selected nucleoids were analyzed from the non-Fpg incubated slides and 80 randomly selected nucleoids were analyzed from the Fpg incubated slides. A total of 160 cells were scored. The net difference (Fpg-incubated minus non Fpg-incubated) is proportional to oxidative DNA damage.

**Statistical Analysis**

Differences between groups for SSB were assessed using the parameter %DNA in tail. Normality of data was tested by the Shapiro-Wilk W-test. Since data did not follow a Gaussian distribution median values were used for each set of cells (16). Data were analyzed by 2-way ANOVA. Means were separated by Tukey’s HSD test (α=0.05). Differences between groups for % Fpg sensitive sites and for % apoptotic cells were assessed by Student’s t-test. Analyses were conducted using the statistical package JMP.
Results

Validation results

The analytical method applied produced good response of linearity in the range of 0.20-7.88 μg mL\(^{-1}\) with correlation coefficient value \(r^2 = 0.998\) (equation, \(y=241932x-4695\)). LOD and LOQ were determined from the calibration plot and were 0.32 μg mL\(^{-1}\) and 0.96 μg mL\(^{-1}\) respectively. Satisfactory results were obtained for all levels with recoveries for low and high concentrations, well above the cut off value of 70%. RSDs % for both low and high concentrations were < 5% therefore acceptable. RSDs % for repeatability and reproducibility were 3.69% and 4.49% respectively. Finally no matrix effect was observed.

Identification of thiram, accumulation in mussel soft tissues and concentration levels in aqueous solutions

The identification of thiram (6.61 min, retention time) was achieved by the LC-ESI-MS system functioning on the positive mode. Selected Ion Monitoring mode (SIM mode) was applied and the characteristic sodium adduct \([\text{M+Na}]^+\) ion of thiram was observed (Figure 1). Accumulation of thiram in mussel soft tissues for exposure of 0.1 mg/L (100 μg/kg) was below the LOD and thus not quantitated. Accumulation for the 1.0 and 10.0 mg/L (1000 and 10000 μg/kg correspondingly) exposure group was 375 μg/kg d.w. and 3115 μg/kg d.w respectively (Table 1). Next step was the determination of the actual concentrations of thiram in aqueous solutions. Thus three different concentration levels were investigated same with those presented in Table 1. Surprisingly for 0.1 and 1 μg mL\(^{-1}\) the only peak which appeared (3.55 min, retention time) and was identified was the peak of another sodium adduct \([\text{C}_3\text{H}_7\text{NS}_2+\text{Na}]^+\) (47). It seems that in more dilute aqueous solutions of thiram the peak of \([\text{C}_3\text{H}_7\text{NS}_2+\text{Na}]^+\) predominates. One possible fragmentation pathway is depicted in Figure 2. The aqueous solution of 10 μg mL\(^{-1}\) contained both sodium adducts \([\text{M+Na}]^+\) and \([\text{C}_3\text{H}_7\text{NS}_2+\text{Na}]^+\) as it is shown in Figure 3. For the sample of 10 μg mL\(^{-1}\), thiram was determined as the sum of areas of peaks which correspond to thiram and its metabolite as equivalent of thiram. The sum concentration measured was 8.19 μg mL\(^{-1}\).

Mussel mortality

The general condition of the animals (i.e. reaction to stimuli, excretion of mucus, attachment to glass-walls) was recorded daily and dead animals were discarded as soon as possible. Profuse mucus secretion was detected only in the high dose groups. Mortalities were low and did not exceed 12.5 % in all exposed groups.

SSB in relation to dose group and tissue type

SSB values were affected by both tissue and dose, while there was significant interaction between dose and tissue (Table 2). Since significant interaction was observed, multiple comparisons were conducted at each tissue for all doses and at each dose for all tissues (Figure 4).

For gill, exposure to 0.1 mg/L thiram caused a statistically significant increase in SSB (see also Figure 5A,B). Higher doses (1.0 and 10.0 mg/L) also caused an increase in relation to control but these were indistinguishable from each other. For haemolymph, exposure to the high dose only (10.00 mg/L) caused an increase in relation to control whereas the low and medium dose did not produce a statistically significant increase in SSB. For digestive gland there was also a dose response with the low dose group being indistinguishable from the control group and the medium and high dose groups being different from the control group but not different from each other.

Oxidative DNA damage in relation to dose group and tissue type

For gill, exposure to 0.1 mg/L caused a very significant increase (P<0.001) in Fpg-sensitive sites which correspond to oxidative DNA damage (see also Figure 5C). For haemolymph and digestive gland, exposure to the medium dose of 1.0...
**Table 1.** Accumulation of thiram in mussel soft tissues.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thiram (μg/kg d.b.w.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>n.d.</td>
</tr>
<tr>
<td>100 μg/kg</td>
<td>n.d.</td>
</tr>
<tr>
<td>1000 μg/kg</td>
<td>375</td>
</tr>
<tr>
<td>10000 μg/kg</td>
<td>3115</td>
</tr>
</tbody>
</table>

n.d.: non detected

**Figure 2.** ESI-MS fragmentation of Thiram.

**Figure 3.** Total Ion Chromatogram (TIC) of Thiram in aqueous solution at 10 μg mL⁻¹. The SIM chromatograms of its Sodium Adduct (m/z 263) and the other identified adduct at m/z 143.

**Table 2.** F-values of the effect of tissue and dose and their interaction on SSB values for *Mytilus galloprovincialis* (2-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>2, 24</td>
<td>234.02</td>
<td>12.32</td>
<td>0.0002</td>
</tr>
<tr>
<td>Dose</td>
<td>3, 24</td>
<td>993.18</td>
<td>52.31</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>tissue/dose</td>
<td>6, 24</td>
<td>128.82</td>
<td>6.78</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

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Figure 4. Percentiles of the values of SSB for each tissue of the 3 mussels in each group. Values followed by different letter differ significantly (Tukey’s HSD test at α=0.05). Capital letters (A, B, C) indicate differences within tissue among concentrations while small letters (a, b, c) indicate differences among tissues within a concentration.
mg/L caused a significant increase in Fpg-sensitive sites (Table 3).

**Apoptotic DNA damage in relation to dose group and tissue type**

For gill and digestive gland, exposure to the medium dose of 1.0 mg/L caused a significant increase in halo cells of similar morphology to the cells observed in the staurosporine-injected mussels. These cells are characteristic of apoptotic DNA damage (see also Figure 5D). For haemolymph, exposure to the medium dose of 1.0 mg/L did not cause a significant increase in halo cells. Staurosporine injection caused a significant increase in apoptotic cells (P<0.001)

Table 3. Percentage of apoptotic cells and of Fpg-sensitive sites in mussels exposed to 1.0 mg/L thiram (*: P<0.05 in relation to control, ** P<0.01, ***P<0.001).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (%)</th>
<th>% Fpg sensitive sites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haemolymph</td>
<td>Gill</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0.25 ± 0.5</td>
</tr>
<tr>
<td>1.0 mg/L</td>
<td>0</td>
<td>2 ± 0.41*</td>
</tr>
</tbody>
</table>
for all cell types in relation to control. Injection and/or DMSO does not cause additional DNA damage as proved by a series of experiments in tissues of *Mytilus edulis* and *Mytilus galloprovincialis* (data not shown).

**Discussion**

Even though thiram is toxic to fish (19), bivalves (*Unio tumidus*) were able to withstand 0.1 mg/L thiram for 3 days without mortalities (15). Taking into account the apparent lack of thiram LC50 for bivalves in bibliography, the aforementioned concentration was used as the lowest one which can elicit biochemical responses. This concentration elicited responses in some of *M. galloprovincialis* tissues as mentioned later in this section.

Usually, the determination of thiram is associated with its decomposition to carbon disulfide (CS$_2$) in acidic medium, followed mainly by spectrometry (5, 12, 38) and head space gas chromatography (1, 34). The drawback of these methods is that they are time consuming and they also lack of selectivity or sensitivity. Another way to detect DTCs is to employ Liquid Chromatography (LC) and capillary electrophoresis (CE) with UV and/or electrochemical detection. Recently Blasco et al. (10) applied a quantitative matrix solid-phase dispersion and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS) method for the simultaneous analysis of DTCs and their degradation products in plants. Among the compounds analyzed was thiram which was detected by both APCI and ESI methods with different corresponding ions each time. This method was also applied here and was able to detect concentration-related differences in the way mussels bioaccumulate thiram.

Even though the concentrations tested were high, only certain signs of morbidity (profuse mucus secretion and delayed reaction to stimuli) were observed. Similarly, high concentrations of agrochemicals have not produced mortalities in bivalves (37).

Thiram is not considered genotoxic in *vivo*, despite being Ames positive (19). *In vitro* alkaline filter elution has also produced positive results (increase in SSB) in rat and human cells (9). The discrepancy between *in vivo* and *in vitro* results for thiram is further mirrored in the experiments conducted by Villani et al. (45), where exposure of mice to the maximum tolerated dose caused a borderline increase in SSB in lymphocytes and no increase in splenocytes. In contrast, concomitant exposure of human lymphocytes to thiram *in vitro* caused a significant increase in SSB. Our results for *Mytilus galloprovincialis* can therefore mostly relate to the *in vitro* outcomes of Bjørge et al. (9) rather than to the *in vivo* ones of Villani et al. (45). Bivalves possess unique physiology and biochemistry quite different from higher organisms. As a result, innocuous substances for higher organisms may be biologically active for bivalves and vice versa.

The effect of thiram (SSB increase) was different in each tissue as shown in Figures 4 and 5 and there was a significant interaction between dose and tissue (Table 2). The route of exposure (water-spiking) may be an important factor for gill susceptibility since gill cells would be the first ones to come into contact with thiram. The direct contact of the large surface area of gills with the diluted pollutants may also contribute to this (3). This difference may also stem from the cell subpopulations: whereas the main gill cell type and the agranular haemocytes of *Mytilus galloprovincialis* were sensitive to the genotoxic effects of benzo[a]pyrene (BaP), the agranular ones which are present at varied concentrations were relatively refractory to these effects (44). Regarding the digestive gland, it is considered to be the main organ of metabolism of organic xenobiotics and the main site of biotransformation enzyme activities (8). As a result a number of reactive intermediates produced may directly attack vicinal digestive gland DNA. Furthermore, the highly condensed chromatin of digestive gland nuclei which creates additional alkali labile sites also contributes to % tail DNA (33). This higher baseline damage may have masked here the effect of thiram, at least in
the low dose group.

Thiram, in the present experiments, has caused a significant increase in oxidative DNA damage as measured through Fpg incubation, which quantifies mainly 8-oxo-dG but also other damaged purines according to Collins et al. (14). This increase was most prominent in gill cells rather than in haemocytes and in digestive gland cells. The metabolic system of bivalves, even though markedly different from the mammalian ones is capable of producing oxidizing intermediates (28) which in their turn affect the cell’s metabolism and components (46). For example, in a similar way to the present results, the common aquatic pollutant BaP which necessitates metabolic activation to redox quinones to exert its toxic action, caused formation of 8-oxo-dG in Mytilus galloprovincialis digestive gland (2, 29).

An oxidative mode of action is not unusual for thiram as it has been shown in other animal models. Any imbalance between prooxidant substances and antioxidant defenses in favor of the prooxidants causes oxidative stress associated with damage to cellular macromolecules (40). Thiram has acted as a potent oxidative agent in the liver of broilers fed with this fungicide. The activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were decreased and this lowering of antioxidant defenses has led to lipid peroxidation in the liver of broilers (27). Thiram has also caused deregulation in activities of key antioxidant enzymes (catalase, GSH-Px, SOD, glutathione reductase) in an in vitro model of V79 Chinese hamster ovary cells (22). These results may be partly explained by glutathione (GSH) depletion caused by thiram (13, 23). GSH offers one of the most efficient non-enzymatic protective mechanisms by its conjugation with electrophilic and/or oxidised components. Thiram however possesses a reactive disulfide bond which may react with thiols of critical cellular proteins such as GSH forming mixed disulfides and other products (23), thus rendering them inactive. Furthermore, the GSH redox cycle offers the reducing equivalents for thiram reduction in the cell, minimizing in this way the regeneration of reduced GSH. In more detail, it is postulated that di-thiocarbamates undergo oxidation by Cu²⁺ ions within the cell to their corresponding thiram disulfides. These intermediates are then reduced by GSH, regenerating the parent compound and oxidized glutathione (11). GSH aberrant metabolism due to thiram was further corroborated in studies in V79 CHC which showed a decrease in total GSH/oxidised GSH ratio (22). Therefore, the pro-oxidant effects of thiram are considered to be indirect and mainly due to the lowering of antioxidant defenses.

Finally, a series of important experiments in aquatic organisms showed that thiram oxidative effects are not peculiar to terrestrial organisms. Namely, incubation of Onchocerca mykiss liver with thiram led to loss or decrease of activity of SOD and GSH-Px respectively (6). Exposure of the mussel Unio tumidus to thiram has also caused decrease of activity of selenium-dependent glutathione peroxidase and glutathione reductase as well as decrease in reduced and oxidised GSH in both gills and digestive gland (15).

Regarding testing for apoptosis, apoptotic cells give a characteristic image of large fan-like tail and small head (ghost cells) in the conventional comet assay. However due to their extensive fragmentation, they may become lost during the electrophoresis step (32). In contrast, omission of the electrophoresis step but retention of alkaline unwinding in the alkaline halo assay depicts successfully the unique morphology of apoptotic cells which present a diffuse, spotted halo and a pin-like head clearly delineated from the halo (39). The assay has here revealed a significant increase in apoptotic cells in gills and digestive glands but no increase in haemolymph when mussels were exposed to 1.00 mg/L thiram (approximately 4 μM) for 48 h. In related bibliography, triclosan (3 nM) caused more extensive damage (approximately 16%) after 48 h exposure to Dreissena polymorpha haemolymph (45). This difference cannot be readily explained, however it may be linked to the apoptotic
potency of the tested chemical, the mussel species used and possibly the lower sensitivity in our experiments (staurosporine positive control also caused less than 16% apoptotic cells). An apoptotic mode of action, as this recorded here for *Mytilus galloprovincialis* gill and digestive glands, has been shown in a variety of animal models for thiram. Disarrangement of redox related mechanisms and disintegration of important thiol-containing proteins eventually led to apoptotic cell death in the V79 CHC cells (22). Thiram has also caused apoptosis in avian lymphocytes (4), in bovine capillary endothelial cells (30) and in PC12 cells, the latter due to massive Ca\(^{2+}\) intracellular influx (24).

Overall, a simple and convenient analytical method for the determination of the concentration of thiram in mussel samples revealed that *M. galloprovincialis* may accumulate this fungicide in a dose-dependent way. This accumulation elicited dose- and tissue-related SSB and it also caused increased 8-oxo-dG levels (in all tissues) and % apoptotic cells (in gill and digestive gland). The two last parameters have also increased in a number of *in vivo* mammalian models. It is possible that a common mechanism (reduction, depletion or disintegration of important thiol-containing proteins) is implicated in both mammalian and non-mammalian thiram toxicity.

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Μελέτη βιοσυσσώρευσης του thiram στους μαλακούς ιστούς του μυδιού Mytilus galloprovincialis

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Περιλήψη Η βιοσυσσώρευση του thiram, ενός χαρακτηριστικού μυκητοκτόνου της κατηγορίας των διθειοκαρβαμιδικών, εξέτασθη όσον αφορά στους μαλακούς ιστούς του μυδιού Mytilus galloprovincialis. Επίσης εξετάσθηκε η επίδραση του thiram στην ακεραιότητα του DNA του Mytilus galloprovincialis, μέσω μια σειράς in vivo εκθέσεων σε 0,1, 1,0 και 10,0 mg thiram/L σε θαλάσσιο νερό για 48 h. Παρατηρήθηκε μια δοσοεξαρτώμενη βιοσυσσώρευση συμπλόκων ιόντων του νατρίου με το thiram μετά το πέρας των εκθέσεων. Αυτή η ταυτοποίηση/ποσοτικοποίηση του thiram και των μεταβολιτών του πραγματοποιήθηκε μέσω Υγρής Χρωματογραφίας Φασματομετρίας Μάζας (LC-MS). Όσον αφορά στην ακεραιότητα του DNA του M. galloprovincialis (παρατηρούμενες μονές αλυσίδες DNA) η εμφάνισή τους ήταν ισχυρά δοσοεξαρτώμενη. Αυτό το αποτέλεσμα επηρεάστηκε εξίσου σημαντικά από τον ιστό υπό εξέταση, όπως ήταν αναμενόμενο, δεδομένης της διαφορετικής ευαισθησίας του κάθε ιστού στο thiram και του μεταβολισμού που λαμβάνει χώρα σε αυτόν. Περαιτέρω αναλύσεις DNA βλαβών λόγω οξειδωτικού στρες και απόπτωσης κατέδειξαν πως α) οξειδωτικό στρες ήταν εμφανές σε όλους τους εξεταζόμενους ιστούς και β) κυτταρικά μορφολογικά σχήματα χαρακτηρίζουν παρευρισκόταν τόσο στα βράγχια όσο και στον πεπτικό αδένα του οργανισμού. Από τα παραπάνω αποτελέσματα μπορούμε να υποθέσουμε πως ανίχνευση μεταξύ των αντιοξειδωτικών/οξειδωτικών μηχανισμών των κυττάρων του M. galloprovincialis, προς όφελος των δεύτερων, καθώς και η έναρξη αποπτωτικών κυτταρικών διεργασιών είναι δυνατόν να αποτελέσουν τον κύριο λόγο βλαβών DNA στον M. galloprovincialis λόγω του thiram. Αντίστοιχοι μηχανισμοί έχει αποδειχθεί ότι ισχύουν για τα θηλαστικά.