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Environmental hazard of yperite released at sea: sublethal toxic effects on fish

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HIGHLIGHTS

- We evaluated the toxicity of yperite to fish through *in vivo* and field studies.
- Fish resulted sensitive targets of yperite toxic effects.
- *In vivo* yperite induces EROD and UGT activities and histological alterations.
- The field study showed multiple detrimental effects in fish from the dumping area.

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ABSTRACT

The aim of this study was to evaluate the potential toxicological effects on fish related to the leakage of yperite from rusted bomb shells dumped at sea. Both *in vivo* and field studies have been performed. As for the *in vivo* experiment, specimen of European eel were subcutaneously injected with 0.015, 0.15 and 1.5 mg/kg of yperite and sacrificed after 24 and 48 h. In the field study, specimen of Conger eel were collected from a dumping site in the Southern Adriatic Sea. The presence/absence of yperite in tissues, genotoxicity, detoxification enzymes, histological alterations and gross abnormalities were investigated. Results of the *in vivo* experiment showed a significant increase of EROD activity at both 24 h and 48 h. UGT activity increased significantly at 48 h post injection. An acute inflammatory response after 24 h in skin layers and muscle was observed, associated to cell degeneration and necrosis after 48 h at the highest dose. On field, comet assay revealed genotoxicity in gills of fish from the dumping site. Specimen from the dumping site showed significantly higher EROD activities compared to controls, deep ulcers and papules on skin together with liver and spleen histopathological lesions.

1. Introduction

Historical events which led to the dumping of ordnance in the world's seas date back to WWI. In those years, many countries started to produce huge amounts of weapons and continued during WWII. At the end of this war, countries had two major problems to cope with: poor storage space in their ordnance depots and huge weapons stockpiles. At least until the seventies, dumping at sea was thus considered the best available solution for these materials and was carried out extensively in many seas [1,2]. Recently, the Scientific Community raised concern about the impact of chemical weapons disposed at sea: unexploded ordnance are in fact in such a quantity to represent an actual hazard for those who work at sea and for benthic ecosystems as well. Rusted bombs leak various harmful chemicals such as warfare agents (CWAs) whose environmental fate and toxicity for marine ecosystems are still not known [3,4]. Hence the evaluation of potential effects of CWAs exposure is necessary to carry out ecological risk assessment and to build up an appropriate remediation strategy for marine dumping areas [5].

Yperite (bis[2-chloroethyl]sulphide) is one of the major components of chemical bombs dumped at sea. This blistering agent has low solubility in water (920 mg/L) and due to its high density (1.27 g/cm^3) it sinks to the seabed once leaked in seawater [6].

Yperite's toxicity has been studied mainly on mammals [7–10] and humans [11,12]. Yperite is a strong carcinogenic alkylating agent [13] which reacts with proteins, DNA, and phospholipids,

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but its most important cytotoxic action arises from DNA alkylation and cross-linking. Even low-dose exposure is likely to generate interstrand DNA cross-links, which are lethal to proliferating cells [14]. Another well-established mechanism is the inactivation of sulfhydryl-containing proteins [15] and peptides such as glutathione (GSH). This has implications for the redox state of the cell internum, leading to lipid peroxidation, membrane damage, calcium imbalance, and cell death [16].

These results justify the general concern related to the presence of yperite in the marine environment and call for the urgent need to increase our knowledge on pathways of toxicity and on sensitive targets of this compound in the benthic environment. To date in fact no data are available concerning yperite toxicity in fish species except for a measured 30 days toxicity threshold of 2 mg/L for some fish species [17].

The aim of the present study is to evaluate the potential toxicological effects related to the leakage of yperite from rusted bombs dumped at sea on benthic fish through an *in vivo* experimental exposure and a field study carried out in a dumping area of the Southern Adriatic Sea [18].

An integrated ecotoxicological approach was applied, combining chemical and biological analysis to investigate: presence/absence of yperite in fish tissues, genotoxicity, profile of detoxification enzymes, histological alterations and gross abnormalities.

2. Methods

2.1. In vivo exposure

The European eel *Anguilla anguilla* was chosen as model species due to its adaptability to laboratory conditions and handling and its high sensitivity to xenobiotics [19]. The species also has similar ecological characteristics to European conger *Conger conger*, which was the sentinel species selected in the field study.

Sixty juvenile eels, with a mean weight of 60 gr and total length of 35 cm, were caught from the Orbetello lagoon (Tuscany). Eels were acclimatized for 7 days in 40 l aerated aquaria at 36‰ salinity and 18 °C temperature prior to the experiment. Fish were divided into five groups of 10 specimen each: one received an subcutaneous injection of 100 μl of corn oil (reference group), while other three groups were injected with 0.015, 0.15 and 1.5 mg/kg of yperite (kindly provided by NBC Joint Logistic Centre) dissolved in corn oil; one group was kept untreated in order to reveal potential effects of corn oil or handling conditions. Injection was preferred to seawater exposure to avoid loss of compound due to hydrolysis and for operator safety. As the compound is highly reactive and rapidly metabolized and excreted, an acute experiment has been preferred. Exposed animals received only a single injection and five fish of each group were sacrificed after 24 h or 48 h by percussive blow to the head and blood was withdrawn from the caudal vein by a heparinysed syringe. Two blood slides for each fish were fixed with absolute methanol for 15 min and dried for micronuclei analysis. Each specimen was analyzed (autoptical analysis), measured and weighted. Muscle and liver were removed and stored respectively at −20 °C and −80 °C prior to chemical and detoxification activities analyses. Samples of skin, liver and kidney were fixed in Bouin's solution and stored in alcohol 80% for histological analysis. The experiment was carried out in accordance to Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2. Dumping area and sampling activity

The area selected for the field study is one of the main dumping sites of the Southern Adriatic Sea. It is a stretch of sea nearly one square nautical mile wide, located 35 nm from the coast of Apulia (Fig. 1). The restriction of fishing activities within the study area as well as its remarkable distance from the coast allow the authors to reasonably exclude the presence of additional anthropic pressures within the study area other than the bombs. The seabed is made by coarse sediments enriched with fine particles. Water temperature at the seabed is about 14 °C. At 200–300 m depth several chemical aerial bombs dating back to WW II were previously detected, filmed and classified [20]. Most of these bombs were loaded with yperite. CWAs were clearly visible both from holes and fractures in the bomb's body and on the surrounding seafloor. Levels of total As and Hg measured in European conger were 146.47 mg/Kg (w.w.) and 1.53 mg/Kg (w.w.) respectively [18].

The reference sampling area (reference site) was located in the southern Tyrrhenian Sea (north of Sicily, off Capo d'Orlando). The coast is characterized by a narrow continental shelf and a very step slope (200 m) with the presence of submarine canyons and valleys at a depth >150 m. Information was collected from local archives and fishermen of the area in order to confirm that ordnance had never been dumped in that area supporting the choice of using this area as Reference site. Furthermore, levels of organochlorines in

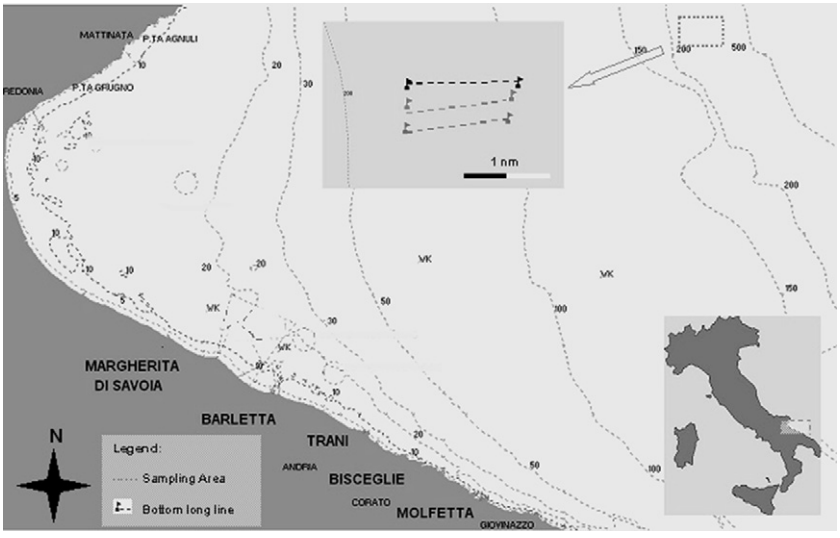

Fig. 1. The dumping site. The dotted rectangle within the bathymetry 200–500 represents the sampling area. Enlarged are the three transects along which were positioned the bottom longlines.

benthic species, were also below the detection limit [21]. As and Hg measured in European conger were 39.99 mg/Kg (w.w.) and 0.67 mg/Kg (w.w.) respectively [18].

Twenty specimens of conger (96.4 ± 21.8 cm tl) were caught in the dumping site, and 8 (95.6 ± 19.1 cm tl) were caught in the reference site, through sea bottom longline. Specimens were individually examined post-mortem to evaluate health status and to detect pathological lesions. A portion of muscle, liver, gills, kidney, intestine and gonad was stored at −20 °C or −80 °C for either chemical or biomarker analysis. Different tissues were fixed in Bouin's solution for histological analysis.

2.3. Chemical analysis

Analysis of yperite in muscles of eel and conger was carried out by the procedure described in Drasch et al. [22]. To test the methodology, a series of experiments were carried out using yperite standard (>90.0%) obtained by distillation of a mixture of yperite–phenyldichloroarsine (Y-FDA) obtained from NBC Joint Logistic Centre demilitarization plants. A 1.27 ppm solution of yperite and dichloromethane (DCM) was prepared while a silica capillary was conditioned by flushing with 12 ml of DCM; 1 ml of solution was injected into the capillary followed by 8 aliquots of 6 ml of solvent (48 ml total). Each aliquot was analyzed by GC/MS. Finally, methanol was passed through the capillary to verify yperite retention by the silica. Samples of fish muscle and liver were spiked with yperite (1 ppm) and extracted with 3 ml of DCM in an ultrasonic bath for 15 min and were then processed as above.

2.4. Micronucleus (MN) test

Blood slices were stained with fluorescence dye DAPI (1:1000 in 0.1 citrate buffer pH 7), selectively able to bind DNA. For each specimen 1000 mature erythrocytes were analyzed under 1000× magnification in order to determine MN frequency [23]. The analysis was made by an Olympus BX60 microscope equipped with UV apparatus for fluorescence relevation at λ 360–400 nm and G365/FT 395/LP420 filter set.

2.5. Single cell gel electrophoresis (SCGE)/comet assay

Only fish from the field study were evaluated (see discussion). The assay was performed on frozen pieces of gill, liver, muscle, kidney, intestine and gonad according to Singh et al. [24]. The parameter selected for quantification of DNA damage was Tail DNA % (*i.e.*, Tail % DNA = 100 − Head % DNA).

2.6. Detoxification activities

Liver microsomal and citosolic fractions were obtained following the method of Corsi et al. [25]. Microsomal Ethoxiresorufin-*O*-deethylase (EROD) activity was measured through a fluorimetric method [26]. UDP-glucuronosyltransferase (UGT) activity was performed fluorimetrically [27]. Glutathione-*S*-transferase GST activity was measured by a spectrophotometric method [28] modified for microplate readers. 190 μl chlorodinitrobenzene 1 mM (ε = 9.6 mM/cm) dissolved in 0.1 M phosphate buffer (pH 7.42, 18 °C) and 10 μl of reduced glutathione 1.5 mM was added to 20 μl diluted citosolic fractions or homogenizing buffer (reference). Absorbance was measured after 2 min at 340 nm. Total proteins were measured spectrophotometrically using bovine serum albumin as standard (0–0.5 mg/ml) [29].

2.7. Histological analysis

Samples of skin, muscle, liver and kidney were processed by routine histological techniques. Tissues were embedded in paraffin and sectioned at 3–5 μ with a Reichert–Jung rotative microtome. In five fish collected from each site, a quantitative analysis of splenic melano-macrophage centres (MMCs) was further performed [30]. Number and percentage area occupied by MMCs were calculated on a total area of 2.25 mm^2, based on three different optical fields (0.75 mm^2/each), by using an image analyzer (Axio Vision 3.01) with JVC colour video camera input.

2.8. Post-mortem examination

Post mortem evaluation was performed on fish experimentally exposed to yperite and on those collected on field, in order to evaluate their general health status and to detect any lesion potentially related to yperite or other toxic compound. The condition of skin, fins, gills and eyes was evaluated and internal organs were observed *in situ* and after dissection.

Furthermore, on the basis of the presence/absence of lesion occurred in different organs, their characteristics and severity, the Health Assessment Index method (HAI; [31]) was used to distinguish and to quantify the health condition of fish collected in the dumping and reference sites.

2.9. Statistical analysis

All parameters were statistically evaluated. Comparison of data from controls and yperite exposed groups were performed through non parametric Mann–Withney test. To avoid any bias due to different sample sizes 8 observations were randomly extracted among the 20 measurements from the dumping site and compared with ref through Mann–Whitney test. The operation was repeated 10 times. $P < 0.05$ was the significance cut-off.

3. Results

3.1. In vivo study

No mortality was recorded throughout the *in vivo* experiment. As no differences were detected in biological parameters between untreated and corn oil injected groups, eels exposed only to corn oil were taken as control group.

3.1.1. Yperite levels

The limit of yperite detection was 0.05 mg/L in the final solution, determined by quadrupole detector. No evidence of yperite was found in tissues of fish exposed to yperite.

3.1.2. Genotoxicity

MN frequencies measured in both controls and yperite injected fish were all below 1‰.

3.1.3. Detoxification activities

Results concerning hepatic detoxification activities are summarized in Table 1. An increase of EROD activity in fish injected with different concentrations of yperite has been observed after 24 h, although significant only at the lowest concentration (0.015 mg/kg). After 48 h a more marked significant induction has been observed except at the highest concentration (1.5 mg/kg). Phase II activities UGT resulted significantly induced after 48 h at all yperite concentrations. On the contrary, GST did not show any clear pattern of modulation, resulting in a slight reduction at lower yperite dose after 24 and 48 h of exposure; a significant increase

Table 1
Hepatic detoxification activities in eels exposed *in vivo* to yperite. Results are given as mean ± s.e. ($N = 5$).

	EROD pmol/min/mg prot		UGT nmol/min/mg prot		GST nmol/min/mg prot	
Time	24 h	48 h	24 h	48 h	24 h	48 h
Oil	148.5 ± 62.03	245.3 ± 31.01	35.95 ± 3.71	27.16 ± 1.53	209.1 ± 30.31	212.69 ± 13.62
0.015 mg/kg	329.2 ± 67.48*	410.4 ± 41.38*	28.24 ± 7.42	36.00 ± 2.87*	188.6 ± 36.49	182.3 ± 8.22
0.15 mg/kg	233.8 ± 19.58	402.3 ± 60.65*	27.35 ± 2.44	36.87 ± 2.41*	299.4 ± 32.55	187.5 ± 28.56
1 mg/kg	216.84 ± 35.56	262.53 ± 44.43	36.08 ± 8.59	38.65 ± 2.57*	321.0 ± 52.83	370.0 ± 26.37*

The asterisk stands for significant difference ($^*p < 0.05$) compared to controls (corn oil).

was observed after 48 h in fish injected with the highest yperite concentration.

3.1.4. Gross lesions and histology

Eels exposed to the highest yperite dose showed a slight skin hyperpigmentation around the site of injection after 48 h, but no gross lesions were displayed in fish injected with lower yperite concentrations and controls.

Histological analysis showed the presence of focal lesions in skin and muscle limited to the area of injection (Fig. 2). Skin lesions were more evident after 48 h. These included cytoplasmic vacuolization and swelling of club epidermal cells in fish injected with 0.015 or 0.15 mg/kg of yperite, and epidermal detached, discrinia, hyperpigmentation in fish exposed to the highest yperite dose (1.5 mg/kg). The latter also showed focal dermatitis of stratum spongiosum and stratum compactum, with steatitis. Fish exposed to yperite at 0.15 and 1.5 mg/kg further displayed myositis charachetrized by rhabdomyolisis, necrosis and inflammatory cell infiltration. No alterations were identified in other organs and in control fish.

3.2. Field monitoring

Some of the following results have been already described in our previous paper [18].

No evidence of yperite was found in tissues of fish from both sites.

Concerning genotoxicity, MN frequencies measured in fish from dumping and control sites did not reveal any significant difference (Table 2). Preliminary analysis of all the available tissues (gill, liver, muscle, kidney, intestine, gonad) only revealed comet tails in gills. We therefore focused on analyses of this tissue. Congers from the dumping site showed comet tails, indicating significant DNA damage quantified as 28.77% tail DNA. Fish from the reference site showed no signs of DNA damage (Table 2).

Table 2
Genotoxic effects measured as MN frequency and SGCE/comet assay in conger (field study). Results are given as mean ± s.e.

	MN frequency ‰	SGCE % tail DNA
CWAs-impacted site ($N = 20$)	0.85 ± 0.88	28.51 ± 6.19**
Reference site ($N = 8$)	1.00 ± 0.71	0.8 ± 0.05

The asterisk stands for significant difference between sites ($^{**}p < 0.01$).

Table 3
EROD and UGT activities measured in liver of conger (field study). Results are given as mean ± s.e.

	EROD pmol/min/mg prot	UGT nmol/min/mg prot
CWAs-impacted site ($N = 20$)	401.7 ± 168.6**	30.27 ± 7.83
Reference site ($N = 8$)	50.64 ± 17.41	35.09 ± 7.17

Asterisk means significant difference between sites ($^{**}p < 0.01$).

As regards to detoxification activities, a significantly higher EROD activity has been measured in congers from the dumping site respect to specimens from the reference site (Table 3). On the contrary no difference was observed in UGT activity (Table 3).

Regarding gross lesions observed during necroscopy, congers captured at the CWAs site displayed different skin lesions (Fig. 3),

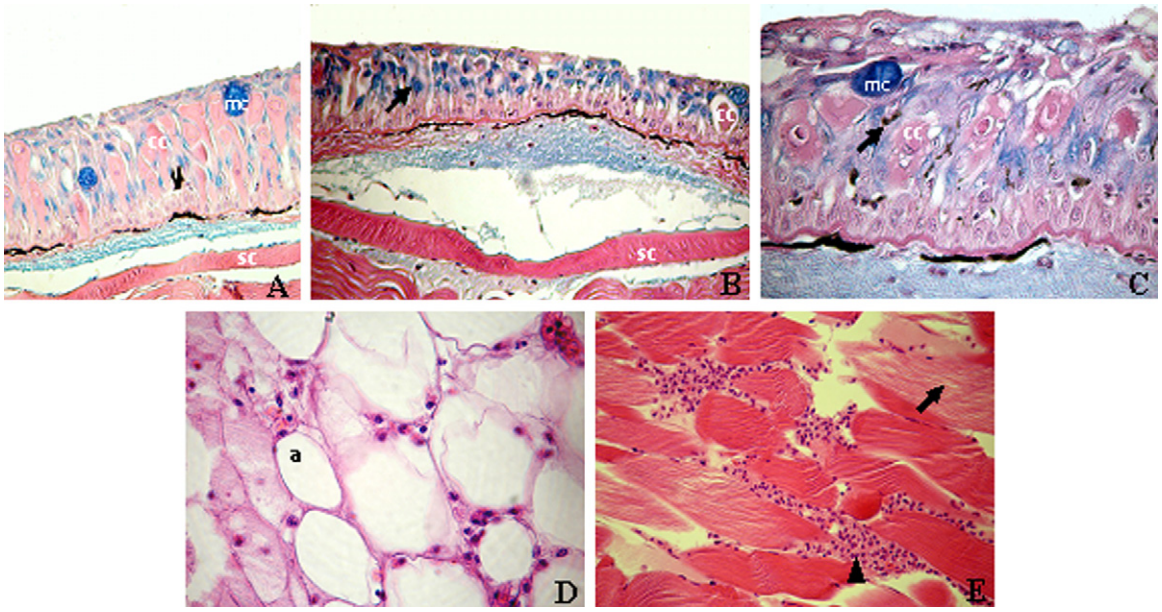

Fig. 2. Skin and muscle alterations in European eel *Anguilla anguilla* 48 h after yperite injection. (A) Control skin (H/E & blue alcian, ×100); (B–C) Epidermal discrinia (arrow), vacuolization of club cells (arrowhead) and hyperpigmentation (arrow) (H/E & blue alcian, ×100 B; ×200 C); (D) Steatitis (H/E, ×200); (E) Myositis with infiltrates of inflammatory cells (arrowhead) and necrosis of muscle (arrow) (H/E, ×100). mc, mucous cells; cc, club cells; sc, scale; a: adipocyte.

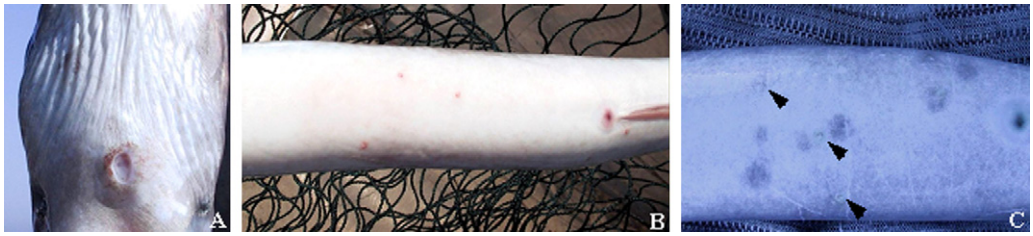

Fig. 3. Skin lesions observed in European conger *Conger conger* captured within the dumping area. (A) Deep ulcer; (B–C) Ulcerated papules and signs of healing (arrowhead).

such multifocal ulcers (diameter 5–10 mm), mostly located close to the mandible and isthmus, and ulcerated papules (diameter 2–3 mm) on lateral and ventral side of the body. The flogosis of the vent and anal fin was further observed in some specimens. Liver nodules and spleen congestion were also observed, together with a severe anisakid nematodes infestation which characterized numerous specimens from CWAs site. Population health index resulted two times higher in specimens from the CWAs site compared to fish from the reference site (62 *vs* 31), indicative of a worst state of health [31].

Histological analysis highlighted the presence of multiple lesions in fish captured in the dumping site (Fig. 4). At the skin level these included moderate to severe dermatitis with vascular endothelial swelling, haemorrhages, dermal oedema and

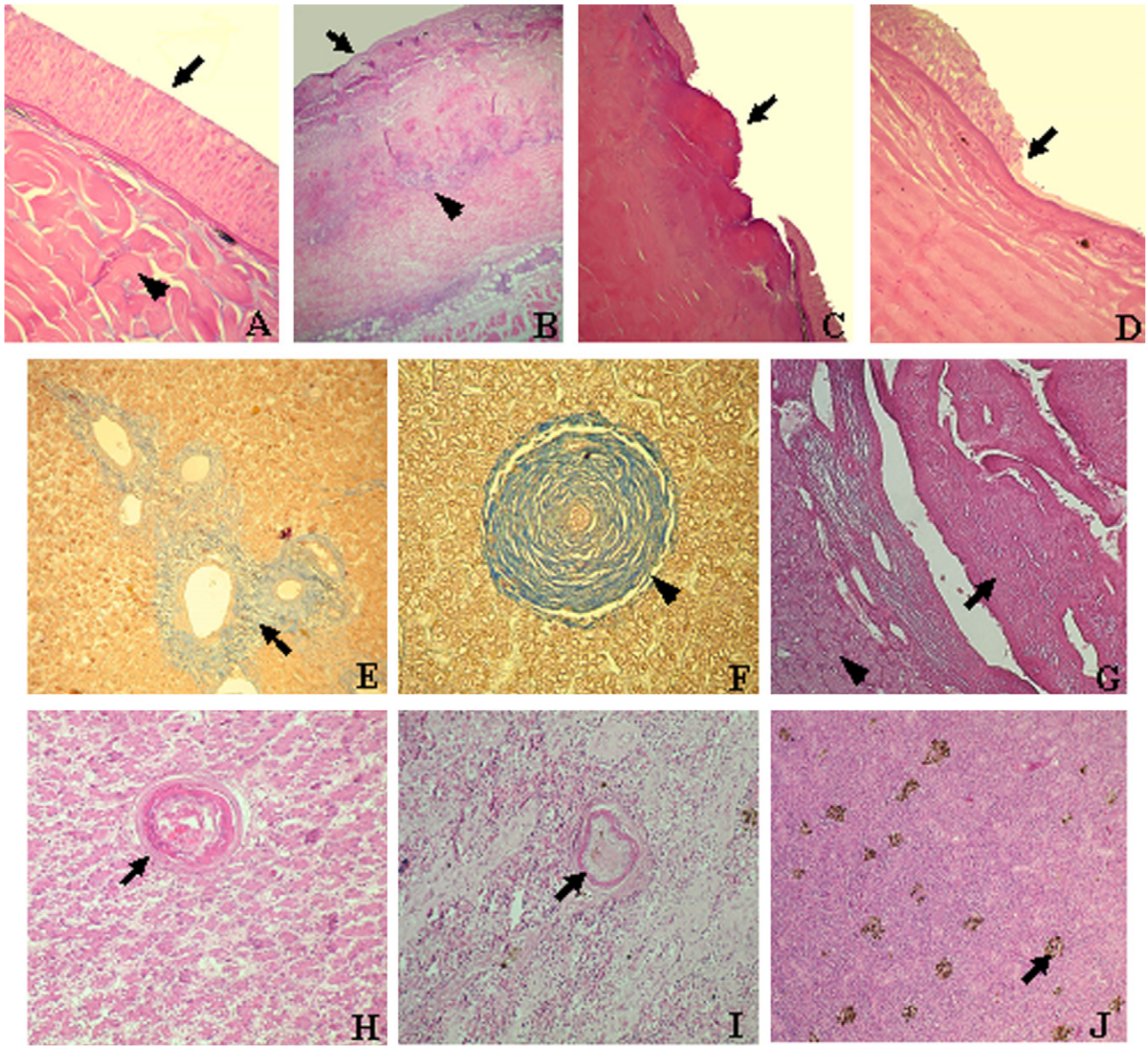

Fig. 4. Histological lesions observed in European conger *Conger conger* captured within the dumping area. (A) Control skin. Epidermis (arrow) and derma (arrowhead) (H/E, ×100); (B–C) Skin ulcer with exfoliation and loss of epidermis (arrow) and intense dermatitis (arrowhead) (H/E, x50 B; ×100 C); (D) Wound healing process (arrow) (H/E, ×100); (E–F) Liver. Periportal (arrow) and bile ducts fibrosis (arrowhead) (Mallory trichrome, ×100 E; ×200 F); (G) Liver neoplasm (arrow) and normal liver tissue (arrowhead) (H/E, ×200); (H–I) Hepatic and splenic granuloma (arrow) (H/E, ×100); (J) Hyperplasia of splenic MMCs (arrow) (H/E, ×100).

inflammatory cell infiltration, exfoliation and necrosis of epidermidis. In liver samples, hyperplasia and fibrosis around the bile ducts, pericholangitis, periportal fibrosis, granulomas and foci of cellular alteration were observed. A liver neoplasm was further observed in a single specimen and is under investigation. Granulomatous lesions and congestion were displayed in spleen samples. In this organ, both the number of MMCs (32 ± 3.9 *vs* 17 ± 3.8) and the percentage of area occupied by MMCs/2.25 mm^2 (1.6 ± 0.3 *vs* 0.7 ± 0.2) resulted significantly higher ($p < 0.05$) in fish from CWAs site in comparison to controls.

4. Discussion

This study investigated the hazard for benthic fish related to the presence of yperite in the marine environment. To this end, results of an experimental *in vivo* exposure and a field monitoring study in a CWAs dumping area were discussed.

Due to the lack of information concerning toxicity of yperite for marine biota the *in vivo* experiment has been performed with the aim to identify potential biological targets of yperite toxicity. Besides the field study aimed to assess the health status of a fish population naturally exposed to yperite. The dumping area selected in the present study represents a unique hot spot of CWAs contamination. Several hydrolysis products of yperite (1,4-thioxane, 1-4-dithiane 1-oxa-4,5-dithiapane and 1,2,5-trithiapane) and high levels of arsenic were detected in sediments collected from this area (up to 44.81 mg/Kg d.w.) compared the reference (4.5 mg/Kg d.w.) [20]. These results suggest a leakage of harmful chemicals (particularly yperite) from rusted bomb shells as well as their potential bioavailability for benthic species inhabiting the area.

Based on the low log Kow of 2.41 and fish Bioconcentration factor of 14.3 [32] yperite is not expected to be persistent in fish tissues such as muscle. On the contrary, adducts with macromolecules (*e.g.* proteins) seem to offer a more persistent biological reservoir (up to months) of this compound [33]. Accordingly, no detectable levels of yperite and its degradation products were found in fish tissues after the *in vivo* exposure.

Histological lesions on skin layers and muscle as well as significant increase of liver detoxification activities seem to highlight the presence of yperite throughout the organism soon after injection. A fast distribution of yperite has been observed in mice following inhalation exposure and in guinea pig following skin exposure [13]. The authors reported also a rapid excretion of the compound (within 48 h), as already observed in mammals and in line with our findings.

In accordance with the results from the *in vivo* study and with the known properties of the compound in the environment, no level of yperite was detected in fish from the dumping area. The use of other tissues such as bile or blood seems more suitable to reveal the presence of yperite and is thus recommended in future studies.

DNA is the most functionally sensitive target of yperite in cells, and the interstrand DNA cross-link produced by bifunctional yperite metabolites is probably the major mechanism of cytotoxicity [12,14]. However MN analysis in eels did not reveal a genotoxic effect due to yperite. The test measures the presence of aberrant DNA division during the anaphase of mitosis or meiosis, with the formation of a micronucleus [34]. MN induction is a cell cycle-dependent phenomenon [35] so this event may have not occurred in cells of our samples within the exposure period. The activation of DNA repair mechanisms or fast removal of apoptotic cells might also reduce the presence of micronucleated cells in the organism leading to underestimation of actual genotoxic effect.

Due to this reason SGCE/comet assay was also performed in fish captured within the dumping area. DNA strand breaks were significantly higher when compared with congers from the reference site, highlighting the potential genotoxic hazard of chemical agents relased from bombs. On the contrary, no difference in MN generation was observed. In light of the above, a potential genotoxic mechanism producing DNA strand breaks instead of cromosomal aberration maybe suggested for yperite. Some authors also suggested the higher sensitivity of comet assay respect to MN [35–38]. Another possible explanation for the observed discrepancy between the two endpoints could be the higher vulnerability of gills cells rather than erythrocytes [39]. Observed genotoxic effects in fact were specific to the respiratory system. Under natural conditions gills are the first target of waterborne pollutants, being the primary site of uptake of substances dissolved in water and having large surface areas in direct contact with noxious substances [40]. Uptake of CWAs through gills is therefore possible.

Results concerning liver detoxification activities indicate that yperite is undergoing active metabolism. Yperite can easily penetrate epithelial tissues due to its lipophilic nature and be rapidly transported through body circulation, causing systemic intoxication besides of the local damaging capacity [41]. Thus liver, being the major site of biotrasformations and clearance, represents an important target of its toxicity [42]. The cytochrome P450 system (CYP450) and particularly the CYP1A EROD enzyme is considered the major enzyme involved in detoxification of dioxin-like pollutants in fish [19]. Glucuronization and glutathione conjugation of xenobiotic or their metabolites are predominant reactions of piscine phase II systems [43]. To date no information is available regarding the effects of yperite and other CWAs on fish detoxification mechanisms, and controversial data have been reported in mammals [44–46].

In this study, the significant increase of EROD activity observed in fish experimentally exposed to yperite suggests the involvement of the CYP1A in detoxification of yperite in fish. EROD activity reached a peak at the lowest concentration followed by a decrease at the highest dose. Such biphasic pattern of induction is often reported in response to environmental pollutants (as dioxins) due to a competitive inhibition of catalytic activity at high inducer concentrations [47]. Another possible explanation could be the inhibition of the enzyme activity by yperite inducing an excess of ROS as reported in rodents [46]. The observed biphasic EROD induction seems to suggest that the activity is more suitable to assess exposure to low yperite concentrations.

The higher UGT activities observed after 48 h of exposure suggest that yperite could be excreted from the body as glucuronide conjugates. On the contrary, a clear involvement of GST in yperite response cannot be predicted on the basis of our results. Based on these results, GST was not measured in fish collected during the field study.

The higher EROD activity observed in fish from the dumping site seems to confirm our findings from the *in vivo* experiment suggesting and active involvement of CYP1A in yperite metabolism in fish. On the contrary no modulation of UGT was observed. This result is in line with previous field studies which failed to observe any alteration in UGT activity in fish resident in contaminated sites [19,48]. In fact induction responses of phase II enzymes are less pronounced than phase I and can be more affected by physiological and environmental factors as well as being less extensively studied in fish [19]. The use of EROD activity is thus recommended as marker of yperite toxicity instead of other detoxification activities.

The acute exposure of eels at the highest yperite dose elicited different lesions including skin hyperpigmentation, dermatitis, steatitis and myositis with macrophage and neutrophil infiltration, degenerative process and necrosis. Those lesions have been also described in humans and mammals exposed to yperite [8,49–53]. Erythema, skin blistering and ulcers are common gross lesions induced by yperite, characterized by skin oedema, vasodilatation,

neutrophil infiltration, together with massive cell death and inflammatory response as histopathological findings [54].

On the contrary, no histological lesions were observed in internal organs during the *in vivo* trial. This discrepancy could be potentially related to several factors such as: short time of exposure, fast metabolism and excretion of yperite in fish, reduction of yperite action due to subcutaneous administration. A recent study in rats showed that damage following yperite exposure increased through percutaneous route in comparison with oral or subcutaneous route, assuming a differential metabolism of yperite at skin level due to the greater number of metabolically active and rapidly dividing cells [55].

As already observed in the *in vivo* exposure, severe epidermal and dermal lesions were detected in congers captured in CWAs site. Skin ulcers observed in fish from the CWAs site were deep and with regular aces and some of them were in phase of healing, excluding traumatic effects due to fishing. Skin ulcers on fish are well-recognized as indicators of polluted or otherwise stressed aquatic environments, but their aetiology could be related to different factors including infectious agents [56,57]. Yperite is reported to be detrimental to the immune system and skin lesions are often slow to heal, increasing the susceptibility to secondary infection [49,55,58,59]. In this study, no microbiological analyses were performed on skin ulcers and the direct or indirect effects of yperite can only be assumed. Nevertheless, previous monitoring studies highlighted congers of the area living inside or under rusting bombs [20], making them easily and potentially in direct contact with yperite [18].

In both studies no blisters were pointed out. However it should be noted that fish do not have a keratin layer over the epidermis [60], therefore the typical blisters caused by yperite exposure could be prevented.

Concerning internal organs, liver and spleen of conger from CWAs site displayed proliferative, degenerative, and inflammatory lesions together with an increase in number and size of splenic melano-macrophages centres (MMCs), consistent to a condition of chronic state of illness and environmental degradation [30,61,62]. Similar lesions were described in other fish species living in areas contaminated by CWAs, including yperite and arsenic compounds [63–66]. Arsenic for instance has detrimental effects on fish health even at low concentrations [67,68]. Taking into account the elevated arsenic levels in sediment and captured fish and that yperite weapons contain high concentrations of arsenics, the chronic effect of this heavy metal on fish health can not be excluded.

5. Conclusions

Results of the present study highlight for the first time the sublethal toxic effects of yperite in marine fish. This compound induces relevant biological alterations such as tissue and cell damaging and increased detoxification activities. Some of the alterations observed under laboratory conditions were also found in fish from dumping site. The lack of detectable levels of yperite or its degradation products in fish do not allow to define a clear cause–effect relationship in natural exposure conditions. However, the biological alterations present in fish population from the dumping site highlight the concern related to the presence of CWAs at sea and recommend more studies on marine biota and ecosystems close to dumping areas.

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References

[1] E.J. Hogendoorn, A chemical weapons atlas, Bull. Atom. Sci. 53 (1997) 35–39.
[2] P.G. Brewer, N. Nakayama, What lies beneath: a plea for complete information, Environ. Sci. Technol. 42 (2008) 1394–1399.
[3] H. Sanderson, M. Thomsen, P. Fauser, P.B. Sørensen, Screening level fish community risk assessment of chemical warfare agents in the Baltic Sea, J. Hazard. Mater. 154 (2008) 846–857.
[4] H. Sanderson, P. Fauser, M. Thomsen, J.B. Larsen, Weight-of-evidence environmental risk assessment of dumped chemical weapons after WWII along the Nord-Stream gas pipeline in the Bornholm Deep, J. Hazard. Mater. 215–216 (215) (2012) 217–220.
[5] H. Sanderson, P. Fauser, M. Thomsen, P. Vanninen, M. Soderstrom, Y. Savin, I. Khalikov, A. Hirvonen, S. Niiranen, T. Missiaen, A. Gress, P. Borodin, N. Medvedeva, Y. Polyak, V. Paka, V. Zhurbas, P. Feller, Environmental hazards of sea-dumped chemical weapons, Environ. Sci. Technol. 44 (2010) 4389–4394.
[6] ATSDR, Toxicological profile for sulphur mustard (update), Public Health Service, US Department of Health and Human Service, Washington DC, 2003.
[7] F. Tanaka, A.M. Dannenberg Jr., K. Higuchi, M. Nakamura, P.J. Pula, T.E. Hugli, R.G. Discipio, D.L. Kreutzer, Chemotactic factors released in culture by intact developing and healing skin lesions produced in rabbits by the irritant sulphur mustard, Inflammation 21 (1997) 251–267.
[8] F.M. Reid, J. Graham, N.A. Niemuth, A.W. Singer, S.J. Janny, J.B. Johnson, Sulfur mustard-induced skin burns in weanling swine evaluated clinically and histopathologically, J. Appl. Toxicol. 20 (2000) S153–S160.
[9] U. Wormser, R. Langenbach, S. Peddada, A. Sintov, B. Brodsky, A. Nyska, Reduced sulfur mustard-induced skin toxicity in cyclooxygenase-2 knockout and celecoxib-treated mice, Toxicol. Appl. Pharmacol. 200 (2004) 40–47.
[10] S. Dachir, M. Cohen, E. Fishbeine, R. Sahar, R. Brandies, V. Horwitz, T. Kadar, Characterization of acute and long-term sulfur mustard-induced skin injuries in hairless guinea-pigs using non-invasive methods, Skin Res. Technol. 16 (2010) 114–124.
[11] I.J. Hattersley, J. Jenner, C. Dalton, R.P. Chilcott, J.S. Graham, The skin reservoir of sulphur mustard, Toxicol. In Vitro 22 (2008) 1539–1546.
[12] A.T. Black, P.J. Hayden, R.P. Casillas, D.E. Heck, D.R. Gerecke, P.J. Sinko, D.L. Laskin, J.D. Laskin, Expression of proliferative and inflammatory markers in a full-thickness human skin equivalent following exposure to the model sulfur mustard vesicant, 2-chloroethyl ethyl sulphide, Toxicol. Appl. Pharmacol. 249 (2010) 178–187.
[13] J.M. Benson, B.M. Tibbetts, W.M. Weber, G.R. Grotendorst, Uptake, tissue distribution, and excretion of 14C-sulfur mustard vapor following inhalation in F344 rats and cutaneous exposure in hairless guinea pigs, J. Toxicol. Environ. Health A 74 (2011) 875–885.
[14] K. Ghabili, P.S. Agutter, M. Ghanei, K. Ansarin, Y. Panahi, M.M. Shoja, Sulfur mustard toxicity: history, chemistry, pharmacokinetics, and pharmacodynamics, Crit. Rev. Toxicol. 41 (2011) 384–403.
[15] M.P. Byrne, C.A. Broomfield, W.E. Stites, Mustard gas crosslinking of proteins through preferential alkylation of cysteines, J. Protein Chem. 15 (1996) 131–136.
[16] A.M. Langford, M.J. Hobbs, D.G. Upshall, P.G. Blain, F.M. Williams, The effect of sulphur mustard on glutathione levels in rat lung slices and the influence of treatment with arylthiols and cysteine esters, Hum. Exp. Toxicol. 15 (1996) 619–624.
[17] N.B. Munro, S.S. Talmage, G.D. Griffin, L.C. Waters, A.P. Watson, J.F. King, V. Hauschild, The sources fate, and toxicity of chemical warfare agent degradation products, Environ. Health Persp. 107 (1999) 933–974.
[18] C. Della Torre, T. Petochi, I. Corsi, M.M. Dinardo, D. Baroni, L. Alcaro, S. Focardi, A. Tursi, G. Marino, A. Frigeri, E. Amato, DNA damage, severe organ lesions and high muscle levels of As and Hg in two benthic fish species from a chemical warfare agent dumping site in the Mediterranean Sea, Sci. Total Environ. 408 (2010) 2136–2145.
[19] R. van der Oost, J. Beyer, N.P.E. Vermeulen, Fish bioaccumulation and biomarkers in environmental risk assessment: a review, Environ. Toxicol. Pharmacol. 13 (2003) 57–149.
[20] E. Amato, L. Alcaro, I. Corsi, C. Della Torre, C. Farchi, S. Focardi, G. Marino, A. Tursi, An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea), Mar. Biol. 49 (2006) 17–23.
[21] F. Andaloro, Report Studio della surf-zone in Tirreno meridionale attraverso l'analisi delle relazioni trofiche tra benthos e comunità ittica, 2008, pp. 119.
[22] G. Drasch, E. Kretschmer, G. Kauert, L. Von Meyer, Concentrations of mustard gas bis-2-chloroethylsulfide in the tissue of a victim of a vesicant exposure, JFSCA 32 (1987) 1788–1793.
[23] K. Al-Sabti, C.D. Metcalfe, Fish micronuclei for assessing genotoxicity in water, Mutat. Res. 343 (1995) 121–135.
[24] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell. Res. 175 (1988) 184–191.

[25] I. Corsi, M. Mariottini, C. Sensini, L. Lancini, S. Focardi, P450 Cytochrome, Acetylcholinesterase and gonadal histology for evaluating contaminant exposure levels in fishes from a highly eutrophic brackish ecosystem: the Orbetello Lagoon, Italy, Mar. Pollut. Bull. 46 (2003) 203–212.
[26] M.D. Burke, R.T. Mayer, Ethoxyresorufin: direct fluorimetric assay of a microsomal-O-dealkylation which is preferentially inducible by 3-methylcholantrene, Drug Metab. Dispos. 2 (1974) 583–588.
[27] A.C. Collier, M.D. Tingle, J.A. Keelan, J.W. Paxton, M.D. Mitchell, A highly sensitive fluorescent microplate method for the determination of UDP-glucoronosyl transferase activity in tissues and placental cell lines, Drug Metab. Dispos. 28 (2000) 1184–1186.
[28] W.H. Habig, M.J. Pabst, W.B. Jakoby, Glutathione S-transferases., The first enzymatic step in mercapturic acid formation, J. Biol. Chem. 249 (1974) 7130–7139.
[29] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analyt. Biochem. 72 (1976) 248–254.
[30] J.W. Fournie, J.K. Summers, L.A. Courtney, V.D. Engle, V.S. Blazer, Utility of splenic macrophage aggregates as an indicator of fish exposure to degraded environments, J. Aquat. An. Health 13 (2001) 105–116.
[31] S.M. Adams, A.M. Brown, R.W. Goede, A quantitative health assessment index for rapid evaluation of fish condition in the field, Trans. Am. Fish. Soc. 122 (1993) 63–73.
[32] H. Sanderson, M. Thomsen, P. Fauser, P.B. Sørensen, PBT screening profile of chemical warfare agents (CWAs), J. Hazard. Mater. 148 (2007) 210–215.
[33] D. Noort, H.P. Benschop, R.M. Black, Biomonitoring of exposure to chemical warfare agents: a review, Toxicol. Appl. Pharmacol. 184 (2002) 116–126.
[34] G. Frenzilli, M. Nigro, B.P. Lyons, The Comet assay for the evaluation of genotoxic impact in aquatic environments, Mutat. Res. 681 (2009) 80–92.
[35] A.N. Jha, Ecotoxicological applications and significance of the comet assay, Mutagenesis 23 (2008) 207–221.
[36] K. Belpaeme, K. Delbeke, L. Zhu, M. Kirsch-Volders, Cytogenetic studies of PCB77 on brown trout (*Salmo trutta fario*) using the micronucleus test and the alkaline comet assay, Mutagenesis 11 (1996) 485–492.
[37] I.Y. Kim, C.K. Hyun, Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms, Ecotoxicol. Environ. Saf. 64 (2006) 288–297.
[38] F. Mouchet, L. Gauthier, M. Baudrimont, P. Gonzalez, C. Mailhes, V. Ferrier, A. Devaux, Comparative evaluation of the toxicity and genotoxicity of cadmium in amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*) using the comet assay and the micronucleus test, Environ. Toxicol. 22 (2007) 422–435.
[39] Y. Deguchi, T. Toyoizumi, S. Masuda, A. Yasuhara, S. Mohri, M. Yamada, Y. Inoue, N. Kinae, Evaluation of mutagenic activities of leachates in landfill sites by micronucleus test and comet assay using goldfish, Mutat. Res. 627 (2007) 178–185.
[40] V. Tkatcheva, H. Hyvärinen, J. Kukkonen, L.P. Ryzhkov, I.J. Holopainen, Toxic effects of mining effluents on fish gills in a subarctic lake system in NW Russia, Ecotoxicol. Environ. Saf. 57 (2004) 278–289.
[41] K. Kehe, L. Szinicz, Medical aspects of sulphur mustard poisoning, Toxicology 214 (2005) 198–209.
[42] R.S. Chauhan, L.V.R. Murthy, Effect of topically applied sulphur mustard on guinea pig liver, J. Appl. Toxicol. 17 (1997) 415–419.
[43] S.G. George, Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish, in: D.C. Malins, G.K. Ostrander (Eds.), Aquatic Toxicology, Molecular, Biochemical, and Cellular Perspectives, Lewis Publishers, Boca Raton, Florida, 1994, pp. 37–85.
[44] F. Pons, J.H. Calvet, M. Haag, V. Raeppel, T. Keravis, N. Frossard, Altered expression of lung cytochrome P450 3A1 in rat after exposure to sulfur mustard, Pharm. Technol. 88 (2001) 40–44.
[45] E. Hodgson, R.L. Rose, Pesticide metabolism and potential for metabolic interactions, J. Biochem. Mol. Toxicol. 19 (2005) 276–277.
[46] J.P. Gray, V. Mishin, D.E. Heck, D.L. Laskin, J.D. Laskin, Inhibition of NADPH cytochrome P450 reductase by the model sulfur mustard vesicant 2-chloroethyl ethyl sulfide is associated with increased production of reactive oxygen species, Toxicol. Appl. Pharmacol. 247 (2010) 76–82.
[47] J.R. Petrulis, N.G. Bunce, Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-*O*-deethylase (EROD) assay to estimate exposure to dioxin-like compounds, Toxicol. Lett. 105 (2000) 251–260.
[48] C. Della Torre, I. Corsi, G. Perra, F. Nardi, S. Focardi, Phase I. and III drug-metabolizing enzymes are sensitive indicators of petroleum hydrocarbon contamination in the red mullet (*Mullus barbatus*), Mar. Environ. Res. 70 (2010) 95–101.
[49] V. Paromov, Z. Suntres, M. Smith, W.L. Stone, Sulfur mustard toxicity following dermal exposure role of oxidative stress, and antioxidant therapy, J. Burns Wounds 7 (2007) 1–7.
[50] N. Tewari-Singh, S. Rana, M. Gu, A. Pal, D.J. Orlicky, C.W. White, R. Agarwal, Inflammatory biomarkers of sulfur mustard analog 2-chloroethyl ethyl sulfide-induced skin injury in SKH-1 hairless mice, Toxicol. Sci. 108 (2009) 194–206.
[51] M. Ghanei, Z. Poursaleh, A.A. Harandi, S.E. Emadi, S.N. Emadi, Acute and chronic effects of sulfur mustard on the skin: a comprehensive review, Cutan. Ocul. Toxicol. 29 (2010) 269–277.
[52] J.M. Benson, J.C. Seagrave, W.M. Weber, C.D. Santistevan, G.R. Grotendorst, G.S. Schultz, T.H. March, Time course of lesion development in the hairless guinea-pig model of sulfur mustard-induced dermal injury, Wound. Rep. Reg. 19 (2011) 348–357.
[53] L.B. Joseph, D.R. Gerecke, D.E. Heck, A.T. Black, P.J. Sinko, J.A. Cervelli, R.P. Casillas, M.C. Babin, D.L. Laskin, J.D. Laskin, Structural changes in the skin of hairless mice following exposure to sulfur mustard correlate with inflammation and DNA damage, Exp. Mol. Pathol. 91 (2011) 515–527.
[54] K. Kehe, F. Balszuweit, D. Steinritz, H. Thiermann, Molecular toxicology of sulphur mustard-induced cutaneous inflammation, Toxicology 263 (2009) 12–19.
[55] R. Vijayaraghavan, A. Kulkarni, S.C. Pant, P. Kumar, P.V. Rao, N. Gupta, A. Gautam, K. Ganesan, Differential toxicity of sulfur mustrad administered through percutaneous subcutaneous, and oral routes, Toxicol. Appl. Pharmacol. 202 (2005) 180–188.
[56] E.J. Noga, Skin ulcers in fish: *Pfiesteria* and other etiologies, Toxicol. Pathol. 28 (2000) 807–823.
[57] M. Law, Differential diagnosis of ulcerative lesions in fish, Environ. Health Perspect. 109 (2001) 681–686.
[58] Z.M. Hassan, M. Ebtekar, Immunological consequence of sulfur mustard exposure, Immunol. Lett. 83 (2002) 151–152.
[59] Z.M. Hassan, M. Ebtekar, M. Ghanei, M. Taghikhani, M.R. Noori Daloii, T. Ghazanfari, Immunobiological consequences of sulfur mustard contamination. Iran, J. Allergy Asthma Immunol. 5 (2006) 101–108.
[60] R.C. Henrikson, A.G. Matoltsy, The fine structure of teleost epidermis. Introduction and filament containing cells, J. Ultrastruc. Res. 21 (1968) 194–212.
[61] D.E. Hinton, H. Segner, T. Braunbeck, Toxic responses of the liver, in: D. Schlenk, W.H. Benson (Eds.), Target Organ Toxicity in Marine and Freshwater Teleosts, vol. 1, Organs, Taylor & Francis Publishers, New York, 2001, pp. 224–268.
[62] D.W.T. Au, The application of histo-cytopathological biomarkers in marine pollution monitoring: a review, Mar. Pollut. Bull. 48 (2004) 817–834.
[63] E.M.B. Sorensen, R.R. Mitchell, C.W. Harlan, J.S. Bell, Cytological changes in fish liver following chronic, environmental arsenic exposure, Bull. Environ. Contam. Toxicol. 25 (1980) 93–99.
[64] E.M.B. Sorensen, N.K.R. Smith, Hemosiderin granules: cytotoxic response to arsenic exposure in channle catfish, Bull. Environ. Contam. Toxicol. 27 (1981) 645–653.
[65] W. Wawrzyniak, E. Grawinski, An attempt diagnosis of etiology and pathogenesis of ulcer and necrotic foci of some Baltic fishes, Acta Ichthyol. Piscat. 21 (1991) 43–58.
[66] N. Kostanis, J. Iliopolou-Georgudaki, Arsenic induced liver hyperplasia and kidney fibrosis in Rainbow trout (*Onkorhynchus mykiss*) by microinjection technique: a sensitive animal bioassay for environmental metal-toxicity, Bull. Environ. Contam. Toxicol. 62 (1999) 169–178.
[67] C.R. Lage, A. Nayak, C.H. Kim, Arsenic ecotoxicology and innate immunity, Integr. Comp. Biol. 46 (2006) 1040–1054.
[68] D.D. Ghosh, D.R. Saha, S. Bhattacharaya, S. Mazumder, Chronic exposure to low concentration of arsenic is immunotoxic to fish: role of head kidney macrophages as biomarkers of arsenic toxicity to *Clarias batrachus*, Aquat. Toxicol. 92 (2009) 86–94.