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ISTITUTO CENTRALE PER LA RICERCA
SCIENTIFICA E TECNOLOGICA APPLICATA AL MARE

R.E.D. C.O.D. Project

RESEARCH ON ENVIRONMENTAL DAMAGE CAUSED BY CHEMICAL ORDNANCE DUMPED AT SEA

Contract n° B4-3070/2003/368585/SUB/D.3




Final Scientific Report

Ezio Amato (Responsible for technical aspects)

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Via di Casalotti, 300 - 00166 Roma • ☏ +39.06.61570455 • Fax +39.06.61561906 /.61550581
http://www.icram.org e-mail: e.amato@icram.org

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ISTITUTO CENTRALE PER LA RICERCA
SCIENTIFICA E TECNOLOGICA APPLICATA AL MARE

Participant Institutions

ICRAM

Istituto Centrale per la Ricerca scientifica e tecnologica Applicata al Mare
Via di Casalotti, 300 00166 Rome, Italy
Dr. Ezio Amato • ☏ +39.06.61570455; fax +39.06.61561906; e-mail e.amato@icram.org

CoNISMa

Consorzio Nazionale Interuniversitario per le Scienze del Mare
Local Unit of Bari - Biology Department of Bari University
Via Orabona, 4 70126 Bari, Italy
Prof. Angelo Tursi • ☏ +39.080.5443351; fax +39.080.5443358; e-mail a.tursi@biologia.uniba.it

Local Unit of Siena - Environmental Science Department "*G. Sarfatti*" of Siena University
Via Mattioli 4, 53100 Siena, Italy
Prof. Silvano Focardi • ☏ +39.0577.232833; e-mail focardi@unisi.it

IBIM-CNR

Istituto di Biomedicina e di Immunologia Molecolare "Alberto Monroy" of Consiglio Nazionale delle Ricerche
Via Ugo La Malfa 153 90146 Palermo, Italy
Dr. Valeria Matranga • ☏ +39.091.6809551; fax +39.091.6809557; e-mail matranga@ibim.cnr.it

CETLI NBC

Centro Tecnico Logistico Interforze NBC
Via Braccianense Claudia Km 67.300, Civitavecchia, Italy
Col. Giacinto Costantino (Director) • ☏ +39.0766.33560

On the cover

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1: Chemical weapons M47A1 transported to Bari harbour by the operators of the Apulian Ports Demilitarisation Centre, 1947-1953 (source: State Archives in Bari)

2: Temporary storage of CWs collected from Molnfalcone harbour, 2005 (source: CETLI)

3: Skin ulcer observed during the autoptic examination of a specimen of *C. Conger* captured in CWs dumping area (Ph. Tommaso petochi, ICRAM)

4: Conventional aerial bomb located on the seafloor off Pianosa Island (Ph. Ezio Amato, ICRAM)

Authors: Ezio Amato, Luigi Alcaro, Ilaria Corsi, Camilla Della Torre, Cristina Farchi, Pierpaolo Giordano, Giovanna Marino, Marco Matiddi, Milena Modena, Tommaso Petochi, Michela Pisoni, Valerio Sammarini, Marilena Di Nardo, Cap.Vincenzo Ricci, Col. Antonello Massaro, Davide Baroni

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1 Summary

Useless, obsolete, recovered ammunitions, explosives and war material of any sort have been dumped in oceans and seas for decades. Among the military ordnance dumped at sea, chemical weapons (CWs) and riot control agents represent a not negligible percentage, also in the Mediterranean Sea. The yearly rate of dumping is slowly diminuishing also because of the entry into force of international conventions (i.e. the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction, Paris 1992 and the 1996 Protocol to the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter, 1972) stimulating the adoption of other disposal practices. Howhewer, the actual amount of dumped war material, subject to the corrosive action of sea water which causes the release of chemical products, has to be considered as a relevant source of persistent pollutants in need of in-depth scientific investigations.

Taking into consideration the extension of the dumping sites, the wide dispersion of the dumped war material due to trawling fishery, their increasingly rusted conditions and a number of other factors, there is a need for data and information suitable to provide a sound scientific base to carefully evaluate costs and benefits deriving from possible clean up activities at sea.

At present, worldwide scientific studies regarding the ecological effects of dumped ordnance are scarce and often provide only partial and uncertain results. This topic, in fact, has been considered in international *fora* only in the recent past and objective difficulties in carrying out on field and laboratory operations in safety conditions for the personnel require that *ad hoc* safety systems and infrastructure must be set up beforehand[1].

Within the Southern Adriatic Sea, after WW II, both chemical and conventional ordnance, resulting mainly from clean-up activities carried out in bombed harbours and from stockpiles and productions units, were regularly dumped at sea, leading to a significant presence of war material on stretches of the seabed, particularly in the sensitive and less studied bathyal habitats.

[1] Cfr.: Muribi M., 1997. Toxicity of mustard gas and two arsenic based chemical warfare agents on Daphnia magna for the evaluation of the ecotoxicological risk of the dumped chemical warfare agents in the Baltic Sea. Fœrscarets Forskningsanstalt, Umea (Sweden). Avedelningen fœr NBC Skydd. Report n. FOA-R-7-430-222-SE, 33 pp.

The RED COD project aims at achieving a better knowledge on quantities, location and type of ordnance dumped in the Southern Adriatic Sea and at assessing their environmental noxiousness and possible clean up activities by means of the best available technologies.

Several actions have been carried out by the Partners of the project, starting from the acquisition of historical records and scientific and technical papers, through interviewes with local fisheries stakeholders, the mapping of ordnance dumping sites, the carrying out of sampling campaigns and laboratory tests and analyses.

With the aim of assessing the environmental threats of both chemical and conventional ordnance dumped in the Southern Adriatic Sea, two studies, centered within the Tremiti islands archipelago and off shore the harbour of Molfetta, have been carried out in parallel. These sites are already known to represent relevant dumping sites for conventional and chemical war material respectively. Added to that, in order to acquire more information regarding mustard gas and TNT toxicity mechanisms and the biochemical alterations occurring in aquatic fish species after exposure to these compounds and their degradation products, two *in vivo* experiments were performed by exposing, under controlled laboratory conditions, specimens of the European eel *Anguilla anguilla* to different concentrations of mustard gas and TNT respectively along different lenghts of time.

As for the *in situ* study, specimens of *Conger conger* and *Helicolenus dactylopterus* were collected within the dumping sites and in control stations and samples of their tissues have been analysed through a multidisciplinary approach in order to assess noxious effects possibly related to the dumped ordnance. Although neither Chemical Warfare Agents (CWAs) nor TNT and their degradation products were detected within the analysed tissues, the results of other analyses performed provide a rather worrying picture of the affected ecosystems.

Biomarkers analyses highlight a significant difference between specimens collected within the study areas and the reference sites. Particularly the EROD activity is significantly higher for specimens of the CWAs impacted site, thus indicating a clear response of the tested organisms to stressing environmental conditions. Within the same site also As and Hg values in biological samples are higher than the ones measured in reference samples and, in both cases, conger eel turned out to be the most suitable bioindicator providing more clear and solid results.

The results of the Comet essay show that specimens of *Conger conger* captured in the CWs dumping area display a consistent DNA damage in the gills indicating that chemical agents determine their genotoxic effects especially at the breathing apparatus level.

Finally, also histopathological analyses performed on fish captured in the study area off shore Molfetta indicate a poor health status of target species in comparison with control specimens.

The study on TNT highlights as well the presence of a source of contamination confirmed above all by a significant increase of protein Hsp 70 in coelomocytes of the sea urchin *Paracentrotus lividus* and a reduction of hepatic P450 enzyme activities (in particular EROD activity) in *C. conger*.

The analytical results obtained, along with the scientific evidences provided so far in literature, suggest the need for remediation plans to minimize the adverse effects. Taking into consideration the extension of the dumping sites, the occurrence of a wide dispersion of the dumped war material due to trawling activities, their increasingly rusted conditions and a number of other factors, the present report highlights the need to carefully evaluate costs and benefits deriving from possible clean up activities and provides the reader with some hypothesis for sites remediation for both chemical and conventional ordnance dumped at sea.

2 Objectives

The objective of R.E.D. C.O.D. project is to collect the relevant information on environmental and historical aspects concerning dumped chemical and conventional ammunition. The final aim is to achieve a clear overview of the environmental status of the sea areas affected by the dumping of war material. This research has been carried out taking into consideration all the previous results and the experience already developed in the field by the participants to the project.

In particular, the main objectives of the project are:

- to assess the environmental noxiousness of ordnance which are present on the seabed of the Southern Adriatic Sea;
- to achieve a better knowledge on both quantities and type of ordnance dumped in the Southern Adriatic Sea;
- to achieve a better knowledge on the best available technologies for clean up operations

Within the R.E.D. C.O.D. project major relevance has been given to the environmental aspects related to conventional and chemical weapons dumped at sea. For this reason, sampling activities and laboratory analyses of bentho-nektonic fauna associated with the munitions dumping areas have been carried out. Furthermore, a bibliographical research on dumped chemical ordnance and the relevant dumping sites has been performed. Finally, particular care has been dedicated to the identification of the Best Available Technologies (BAT) which could be considered for future possible clean up operations.

The results obtained, as well as the working procedures and protocols developed to carry out both on field and laboratory activities, could be utilised in similar future studies, not necessarily within the Southern Adriatic area alone.

3 Participants

Researchers and Technicians of all the Institutes involved have contributed to the outcome of the activities which are described hereafter.

ICRAM (*Istituto Centrale per la Ricerca scientifica e tecnologica Applicata al Mare*) participated in the following activities:

- coordination of the activities;
- bibliographical, archival researches and interviews with fishery stakeholders in order to chart the main chemical weapons (CW) dumping sites in the Southern Adriatic Sea. Data on quantity and type of ordnance dumped at sea were also gathered;
- sampling campaigns, in collaboration with Conisma, carried out both in the study areas and in the reference sites;
- laboratory analyses, in particular histopathology and micronuclei assays on tissue samples;
- realisation of the documentary "RED COD project: a submerged stockpile"

Conisma (*Consorzio Nazionale Interuniversitario per le Scienze del Mare*), in particular the local unit of Bari[2], organised the sampling campaigns logistics and participated in the sampling activities. Analyses related to DNA damage in fish were also carried out. The local unit of Siena[3] was responsible for laboratory activities related to the stress indexes evaluation and heavy metals contents in fish tissues.

IBIM-CNR (*Istituto di Biomedicina e di Immunologia Molecolare "Alberto Monroy" del Consiglio Nazionale delle Ricerche*) participated in the field activities at the Tremiti archipelago (Southern Adriatic Sea) and developed the laboratory analyses for protein alterations in sea urchin's tissues.

[2] Biology Department of Bari University
[3] Environmental Science Department "*G. Sarfatti*" of Siena University

CETLI (*Centro Tecnico Logistico Interforze NBC*) carried out the analyses related to the contents of chemical agents and explosives compounds as well as their degradation products in fish tissues.

We are very thankful to the Italian Association "*Il mare accanto a noi*" of *Forlì*, which took care of the logistics for the activities carried out on the Pianosa island (Tremiti archipelago), to the Harbour-office Commander of Manfredonia and the Director of Gargano National Park, who provided us with the relevant authorisations in order to operate within the Marine Protected Area of the Tremiti archipelago.

Tab 3–1: Personnel involved

ICRAM	
Ezio Amato	Senior scientist
Luigi Alcaro	Researcher
Antonella Ausili	Senior scientist
Camilla Della Torre	Researcher
Cristina Farchi	Researcher
Pierpaolo Giordano	Technician
Vito Antonio Leuzzi	Researcher
Luigi Manzueto	Technician
Giovanna Marino	Researcher
Marco Matiddi	Researcher
Annalisa Pinsino	Researcher
Valerio Sammarini	Researcher
Enrico Tarulli	Technician
Tommaso Petochi	Researcher

CoNISMa	
Silvano Focardi	Professor
Angelo Tursi	Professor
Antonio Frigeri	Professor
Ilaria Corsi	Researcher
Francesco Mentesana	Technician
Michele Pansa	Technician
Michela Pisoni	Researcher
Marzia Umani	Researcher
M. Pastore Sicuranza	Technician
M. M. Di Nardo	Technician
D. Potenza	Technician

Centro Tecnico Logistico Interforze NBC	
Antonello Massaro	Official
Luca Pinciarelli	Official
Vincenzo Ricci	Official

IBIM-CNR	
Valeria Matranga	Researcher
Caterina Costa	Researcher
Francesca Zito	Researcher

4 Chemical Weapons Dumping Areas

The historical events which led to the dumping of chemical ordnance in the world's seas date back to IWW. In those years, in fact, many countries started to produce significant amount of chemical weapons. The first documented use of chemical agents dates back to the 22nd of April 1915, when the German troops released 180,000 Kg of chloride, previously contained in 5,730 cylinders, along the trenches of Ypres (Belgium). The toxic cloud, which caused thousands of victims among the French and Algerian troops, brought about a 9 Km fracture within the Allied line. During WWI other chemical agents were tested and utilised, mainly yperite, phosgene, diphosgene, chloropicrin, cyanide.

It has been estimated that nearly 125,000 tons of chemical agents were utilised[4]. Some authors [5] believe that 2,5% of the victims were contaminated by yperite.

Although the treaty of Versailles of 1922, signed by Italy, United States, Great Britain, France and Japan, and the 1925 Geneva Convention strictly prohibited any use of chemical weapons, many countries continued to carry out experiments in order to improve both the efficiency of the old chemical agents and to elaborate new sophisticated ones. With regards to Italy, the research activities of the chemical military industry were strengthened. New experimentation and production Centres were established, such as the ones located near Bari and Lecce[6]. The Italian military industry developed the so-called UNCHIM, a mixture of diphosgene, phenyldichloroarsine, chloropicrin and chloroacetophenone; its properties were much more noxious than those of each single component[7]. In 1936, the Italian aircrafts involved in the colonial war in Abyssinia, were equipped with "C500-T" bombs, loaded with yperite, which were utilised successfully to contaminate cultivated fields, to kill the livestock and to put down local unrest. Several operations were performed using these particular bombs[8].

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[4] Mitretek Systems, 2005c. *A short history of chemical warfare during the first world war*. www.mitretek.org/home.nsf/homelandsecurity/WWIChemHistory

[5] Somani S.M., Babu S.R., 1989. *Toxicodynamics of sulphur mustard*. Int. J. Clin. Pharmacol. Ther. Toxicol., 27: 419-435.

[6] State Archives of Italian Army in Rome. *Fondo M7, Busta 530, Fascicolo 1. Documento del 15 marzo 1945 del Ministero della Guerra - Direzione Generale Artiglieria e Motorizzazione*.

[7] State Archives of Italian Army in Rome. *Fondo M7, Busta 530, Fascicolo 1. Documento del 22 dicembre 1938 della Direzione del Servizio Chimico Militare*.

[8] Del Boca. A., 1996. *I gas di Mussolini*. Editori Riuniti

Fig. 4–1 Soldiers of the Italian Airforce around two aerial bombs C-500T loaded with yperite, during the Etiopian War 1936-37 [31]

During WWII countries produced huge quantities of chemical agents and chemical weapons which were transported and stocked along the main war theatres. This huge production was justified by the principle "the right of analogous reprisal", which was set down in the 1925 Geneva Convention.

As World War II drew to a close, the countries had two major problems to cope with poor storage space in their ordnance depots and huge chemical weapons stockpiles overseas. The only possible solution was to dump the weapons at sea.

4.1 World-wide CW dumping sites

Up until at least the seventies, dumping at sea was considered world-wide the best available solution for the disposal of unused armaments and obsolete ordnance.

In many cases the countries producing chemical warfare agents were also responsible for their dumping. In other cases dumping operations concerned CWAs abandoned by other countries at the end of the war. The latter case describes the situation in the Southern Adriatic Sea. The case of the nazi

chemical arsenal is very well known. It was discovered by the Allies at the end of WWII and 296,103 tons of chemical weapons loaded with yperite, fosgene, lewisite and nerve agents were found. In order to deal with their disposal the Allies established a work group called "Continental Committee on Dumping", which had the task of co-ordinating dumping operations. Each country was responsible for the arsenals within its area of responsibility[9]. Besides the Adriatic Sea, dumping operations at sea have commonly been carried out in the Baltic Sea, North Sea, Sea of Japan and the Atlantic Ocean, in particular within the coastal waters of the United States, Ireland and Australia.

On the table 4-1 below the main world-wide sea dumping areas of chemical ammunition are reported. It is necessary to stress that the quantities indicated are only estimated on the basis of scientific reports and that real numbers are often unknown. Moreover, the table indicates only the main dumping sites but several other areas have been utilized for the same purpose. A study carried out by the OSPAR Commission (Convention of the Marine Environment of the north-east Atlantic) highlights nearly 120 potential dumping sites in the north-east Atlantic (for more details see page 16). A review in 2001 concerning world-wide dumping sites used by the U.S. Army for chemical weapons disposal, also mentions the Indian Ocean, the water around Philippines and New Caledonia[10].

[9] Hogendoorn E.J., 1997. *A chemical weapons atlas*. The Bulletin of Atomic Scientists. Vol. **53** (5) (www.bullatomsci.org/issues/1997/so97/so97chepesiuk.html)

[10] US Army Research, Development and Engineering Command, 2001. Off-shore disposal of chemical agents and weapons conducted by the United States. Historical database n° 26. 16 pp. http://www.dailypress.com/news/dp-02761sy0oct30,0,2199000.story

Tab 4–1: Main world-wide sea dumping areas

DUMPING AREA	QUANTITIES OF DUMPED AMMUNITION	SOURCE
BALTIC SEA	The Allies sunk **65,000 tons** of CWs containing mainly yperite and nerve agents	-V. Paka and M. Spiridonov, 2002. http://www.helcom.fi/dps/docs.org
NORTH SEA	USA and UK sunk almost **170.000 tons** of captured German CWs scuttled in old German ships	-HELCOM CHEMU, 1994. http://www.helcom.fi/sea/Reporton chemicalmunitions.pdf
IRISH SEA	It has been estimated over **1,000,000 tons** of munitions have been dumped in the *Beaufort's Dyke.* At least **70,000 weapons** loaded with cyanide, phosgene, nerve agents and yperite	-Hart J., 2000
NORTH AMERICA	Almost **400,000 chemical-filled bombs**, grenades, landmines and rockets have been sunk around the USA coast	-US Army Research, Development and Engineering Command, 2001
AUSTRALIA	About **21,030 tons of chemical weapons** loaded mainly with yperite were dumped off-shore at Queensland, New South Wales and Victoria	-Plunkett G., 2003. www.hydro.gov.au/n2m/dumping/cwa/chemical.pdf
JAPAN	Approximately **4,900 tons of CWAs**, contained in **118,000 shells** and **574,000 canisters**, were dumped at sea	-Kurata H, 1980 -Plunkett G., 2003. www.hydro.gov.au/n2m/dumping/cwa/chemical.pdf
MEDITERRANEAN SEA	It has been estimated that over **1,000,000** munitions have been dumped around the Italian coast. Almost **20,000 chemical weapons**, mainly loaded with Yperite About **3,400 chemical weapons** loaded with yperite and lewisite were dumped off-shore at St. Raphael (France)	-The present project -US Army Research, Development and Engineering Command, 2001

Many of the seas mentioned above have also been theatre to various incidents involving fishermen who have accidentally collected chemical weapons in their trawling nets (Fig. 4–2).[11] [12] [13].

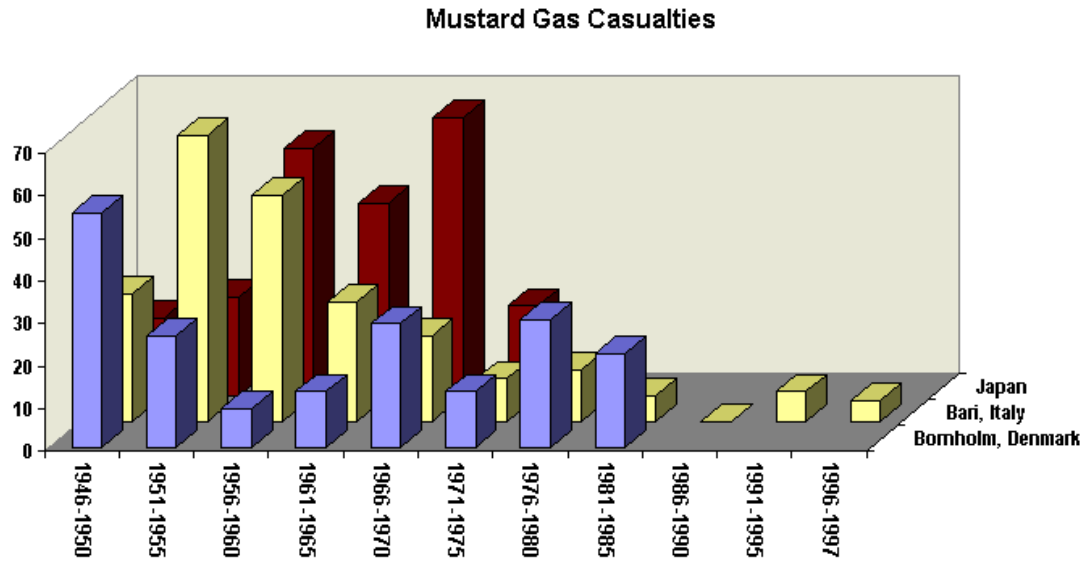

Fig. 4–2 Incidents involving fishermen in the Baltic Sea, Sea of Japan and the Southern Adriatic Sea[14])

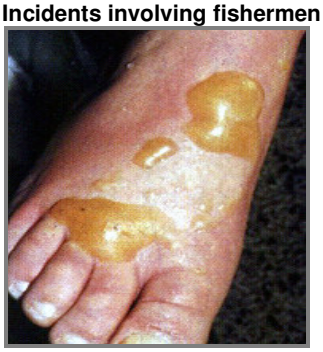

Fig. 4–3 Blister effects on a fisherman's foot caused by contact with mustard gas[15]

Fig. 4–4 Eye irritation involving a fishermen caused by contact whit mustard gas vapours[15]

[11] Assennato G., Ambrosi F., Sivo D., 1997. *Possibili effetti a lungo termine sull'apparato respiratorio della esposizione ad iprite tra pescatori*. La Medicina del Lavoro, **88** n°2.

[12] Aasted A., 1985. Fisherman exposed to Mustard Gas. *Clinical experience assessment of risk of developing cancer*. Ugeskr. Laeg **147** (28): 2213-2215.

[13] Kurata H, 1980. *Lessons learned from the destruction of chemical weapons of the Japanese Imperial forces*. In: Chemical Weapons Destruction and Conversion. Stockolm International Peace Research Institute (SIPRI). Taylor and Francis. London

[14] Mitretek Systems, 2005a. Ocean dumping of chemical weapons. www.mitretek.org/home.nsf/homelandsecurity/OceanDumpChemWeap

4.1.1 Baltic Sea

The Baltic Sea seafloor represents the final destination of many war surplus materials from the German arsenals. The Allies dumped into this sea around 65,000 tons of chemical weapons, mainly close to the Bornholm islands (Denmark), Gotland (Sweden) and near the shallow area commonly known as "Little Belt".

Fig. 4–5 Artillery bombs filled with mustard gas and recovered from the Baltic seafloor[15]

The existence of three wrecks in the vicinity of Bornholm, which are still loaded with at least 11,000 tons of chemical weapons are also very well known[16]. Dumping operations were conducted by the United States, Russia and Great Britain. Due to the low average depth of the Baltic Sea (around 51m), many fishermen have often been victims of incidents caused by the leakage of chemical agents, especially yperite, from the rusted bomb shells accidentally recovered in their trawling nets[17] [18]. The literature also reports five poisoning cases among Danish civilians who were contaminated by yperite while cooking cod eggs. The eggs had probably been soaked in yperite due to their prolonged

[15] Helsinki Commission (HELCOM), 2002. HELCOM *Manual on Co-operation in Response to Marine Pollution within the framework of the Convention on the Protection in the Marine Environment of the Baltic Sea Area (Helsinki Convention)*.**vol. 2.** www.helcom.fi

[16] V. Paka and M. Spiridonov, 2002. *An overview of the research of dumped chemical weapons made by the R/V "Professor Shtokman" in the Gotland, Bornholm & Skagerrak dump sites during 1997-2001*. HELCOM MONAS 4/2002, Document 3/5/INF. http://www.helcom.fi/dps/docs.org

[17] HELCOM CHEMU, 1994. *Report on Chemical Munitions Dumped in the Baltic Sea*, Report to the 16th Meeting of Helsinki Commission, 8 - 11 March 1994 from the Ad Hoc Working Group on Dumped Chemical Munition, Danish Environmental Protection Agency, http://www.helcom.fi/sea/Reportonchemicalmunitions.pdf

[18] NATO/CCMS/NACC -Pilot Study, 1995. *Cross-Border environmental problems emanating from Defence-related installations and activities*. Volume **2**: Chemical Pollution. Final Report. Report n° 205

contact with dirty trawling nets operating within areas affected by the dumping of chemical weapons[19].

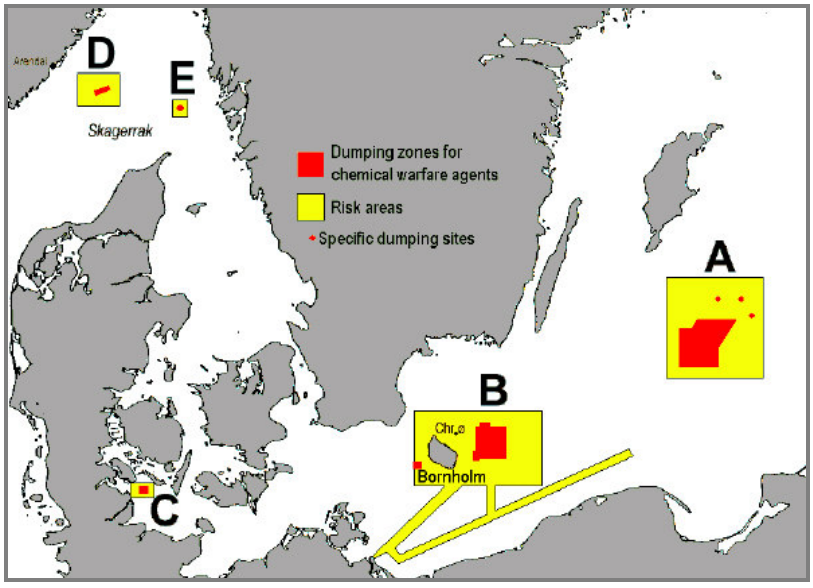

Fig. 4–6 Main Baltic Sea CW dumping sites [15]

4.1.2 North Sea

Between 1946 and 1948, during the operation “Davey Jones Locker", the United States dumped between 30,000 to 40,000 tons of chemical weapons in the Scandinavian area. In following operations, eleven ships loaded with German chemical weapons were dumped into the Skagerrak Strait, in the North Sea, at a depth of 650-1200 m. A similar operation was carried out by Great Britain between 1945 and 1949, when thirty four old merchant ships loaded with 127,000 tons of German ordnance, containing mainly nerve agents [9 17 18] were dumped 25 nm off the coast of Norway.

[19] Hjort N., 1953. *Food poisoning from cod roe contaminated by mustard gas*. Acta Med. Scand., **147** (3): 237-245.

Fig. 4–7 Main North Sea dumping sites of wrecks loaded with chemical weapons

Fig. 4–8 Two aerial chemical weapons scuttled on a wreck in the Skagerrak Strait, observed by a R.O.V. during a survey campaign in 2002 [20]

4.1.3 Irish Sea

Between 1954 and 1956 Great Britain conducted the so-called “Sandcastle” operation, which led to the dumping of 3 old merchant ships 80 nm off the north eastern coast of Ireland. The ships were carrying nearly 70.000 weapons loaded with cyanide, phosgene, nerve agents and yperite, deriving from the British and German arsenals.

[20] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

Fig. 4–9 Ordnance dumping operation overboard in Beaufort's Dyke explosives disposal site

The presence of obsolete ordnance in the area has been confirmed by a survey campaign carried out by the Scottish Office of Agriculture in the *Beaufort's Dyke explosives disposal site* (between the North Channel and the Northern Irish Sea)[21]. By means of a side scan sonar, coupled with a magnetometer, it resulted possible to assess the highest concentration of ordnance in correspondence to the natural trench, 50 km long and 3.5 km wide with an average depth of 200-300m, characteristic of the area. It has been estimated that over 1,000,000 tons of munitions have been dumped in the *Beaufort's Dyke* since the early 1920s[22].

[21] Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD), 1996. *Surveys of the Beaufort's Dyke explosives disposal site, november 1995-july1996.* Fisheries Research Services Report No 15/96. 104pp..

[22] Hart J., 2000. *A review of sea-dumped chemical weapons*. Presented at "The Environment and the common fisheries policy. Threats to and Constraints on Sustainability (Greenmich Forum) 27 january 2000. The Royal Society. 6 Carlton House Terrace, London, Great Britain.

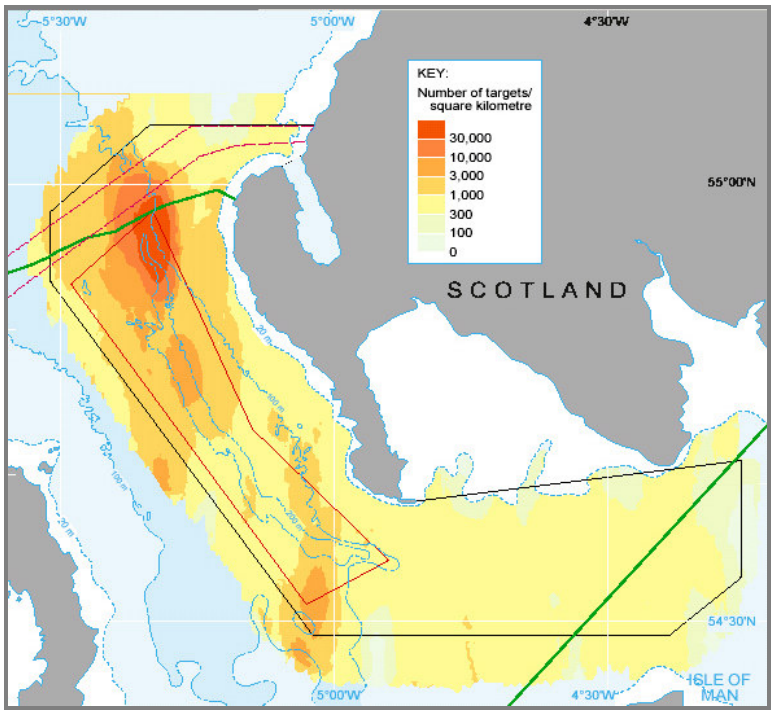

Fig. 4–10 Beaufort's Dyke explosives disposal site. The map shows the distribution of munitions, munitions-related materials and unidentified man-made debris[21]

A study conducted by the OSPAR Commission, concerning the distribution of both chemical and conventional weapons at sea, shows 120 potential dumping sites (Fig. 4–11). This information was supplied by the Contracting Parties on the basis of the "Convention on wide practises and procedures in relation to marine dumped chemical weapons and munitions"[23] [24] [25].

[23] Ospar, 2004. *Convention – wide practises and procedures in relation to marine dumped chemical weapons and munitions*. Biodiversity and dumped material series. www.ospar.org

[24] Ospar, 2004a. *Overview of past dumping at sea of chemical weapons and munitions in the OSPAR maritime area*. Biodiversity series. www.ospar.org

[25] Ospar, 2005. *Overview of past dumping at sea of chemical weapons and munitions in the OSPAR maritime area*. Biodiversity series. www.ospar.org

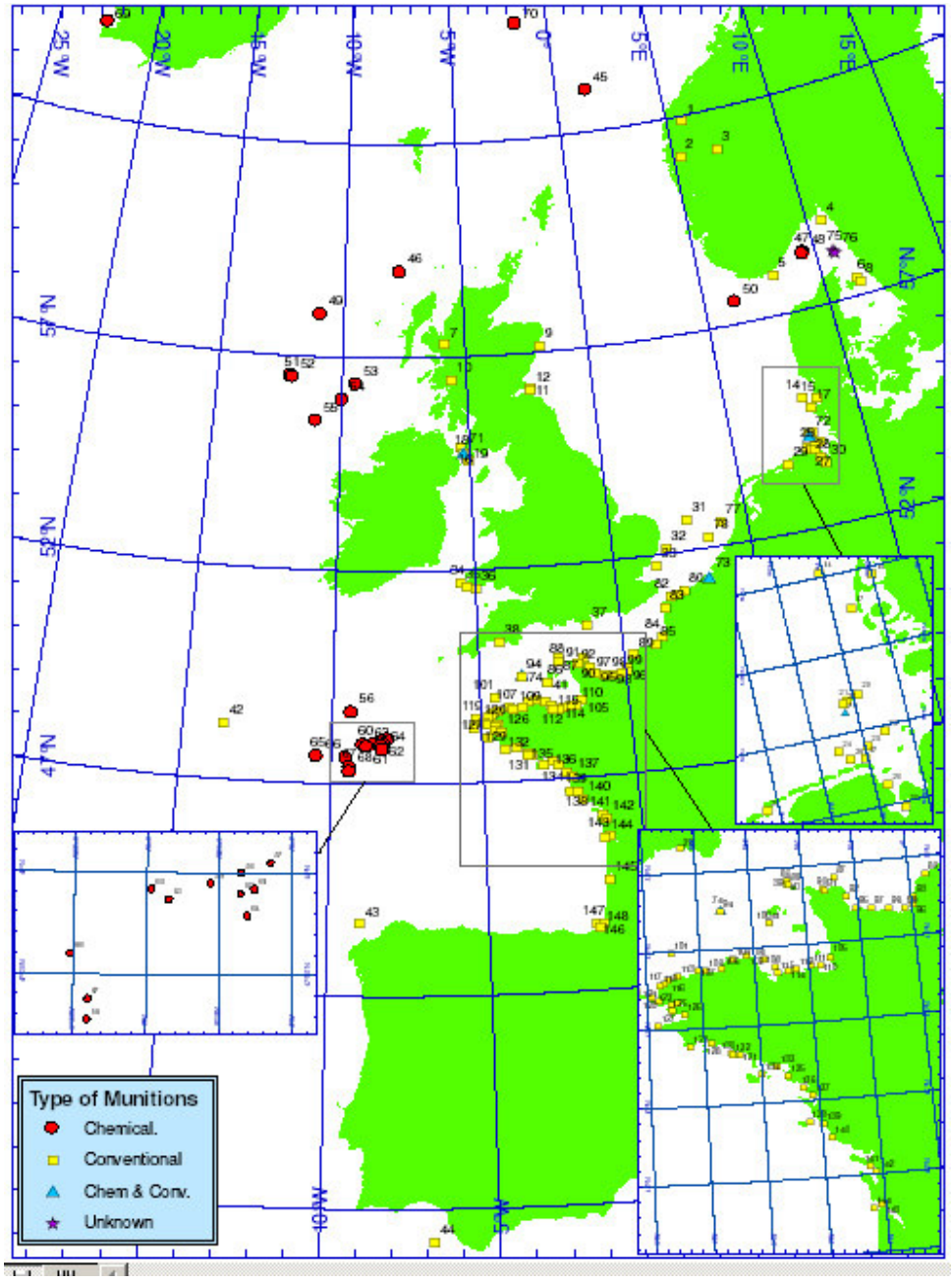

Fig. 4–11 Locations of marine dumped chemical weapons and munitions within the OSPAR Convention Area[25]

4.1.4 North America

Between 1966 and 1967 the United States concluded the operation “Cut Holes and Sink 'Em" (operation CHASE) which aimed to eliminate 51,180 M-55

rockets loaded with sarin (nerve gas) and other chemical weapons. The weapons were loaded on board old ships which were later dumped in open sea.

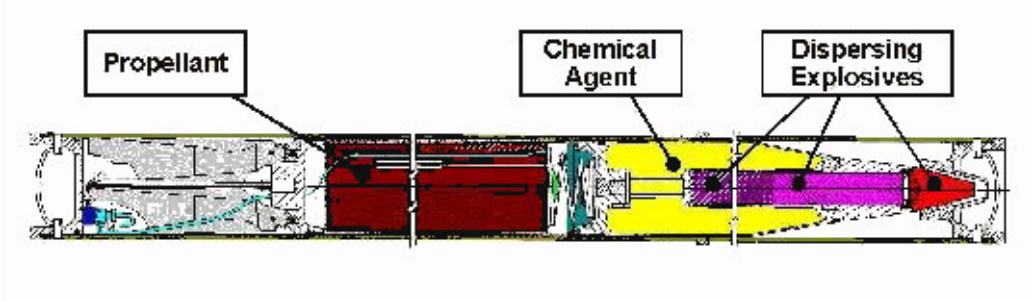

Fig. 4–12 M55Rocket (U.S.A.)[26]

Several dumping activities were carried out by the US Army around the North American coast and nearly 25 dumping sites are known. The main chemical agents involved were yperite, lewisite and nerve agents. 400,000 bombs, grenades, landmines and rockets were loaded with these chemical agents.

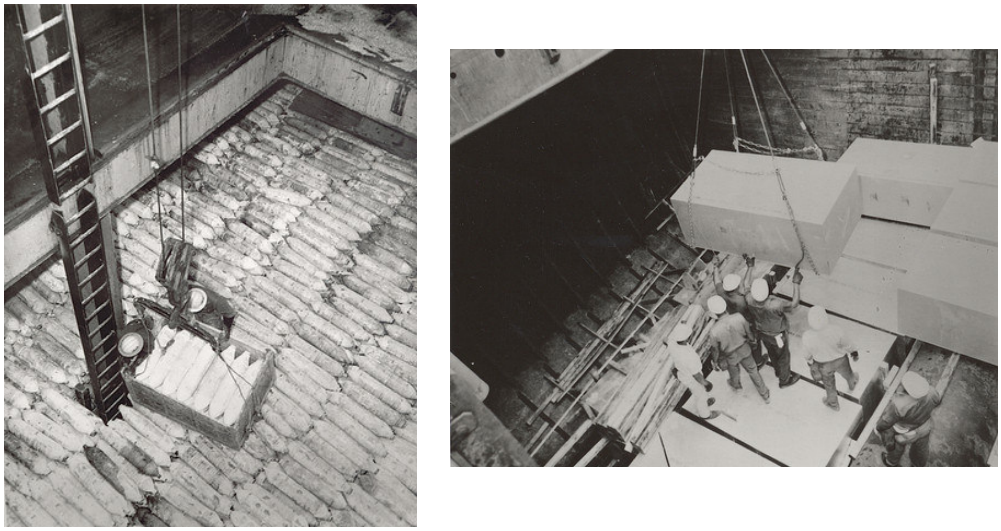

Fig. 4–13 Loading operations of old ships with chemical weapons before sinking in open sea (source Daily News www.dailypress.com/news)

[26] Mitretek Systems, 2005b. Chemical weapons. www.mitretek.org/home.nsf/EnvironmentEnergy/ChemBio#chem

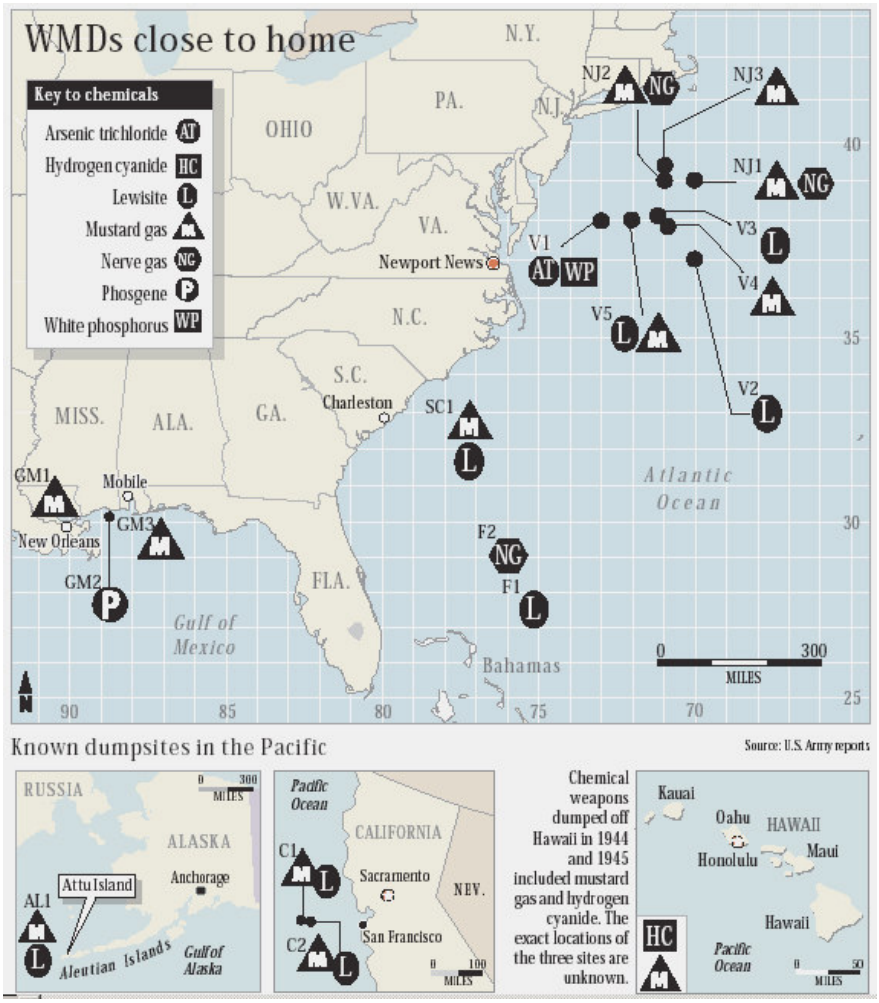

Fig. 4–14 Some of the main CW dumping sites utilised by U.S. Army (source Daily News www.dailypress.com/news)

It has been estimated that the United States have carried out around sixty dumping operations in different areas of the world[27]. These activities were interrupted in 1970 due to the strong opposition of the public opinion towards a new project aimed at eliminating 27.000 tons of CWAs and munitions. In 1972 the "Marine Protection research and Sanctuaries Act", prohibiting any further chemical weapons dumping operations at sea, was finally approved by the Congress (Art. 33, Section 1412)[9]. The last documented dumping operation

[27] US Army Research, Development and Engineering Command, 2001. Off-shore disposal of chemical agents and weapons conducted by the United States. Historical database n° 26. 16 pp. http://www.dailypress.com/news/dp-02761sy0oct30,0,2199000.story

dates back to 1970, when 67 tons of M55 rockets loaded with sarin were dumped off the coast of Florida[28].

4.1.5 Australia

By the end of WWII dumping operations involved Australia as well, where 21.030 tons of chemical weapons were dumped offshore at Queensland, New South Wales and Victoria. The American, English and Australian armies carried out the operations.

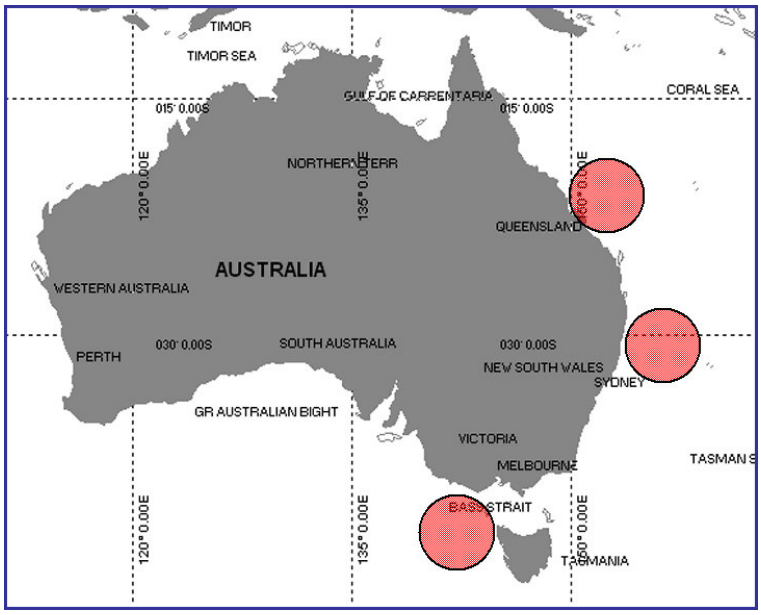

Fig. 4–15 Main dumping sites in Australian territorial waters [29]

Weapons had been kept in Australia in order to be used eventually in the South West Pacific Area. Dumping operations also involved old wrecks loaded with chemical weapons. Yperite was the most common CWA but lewisite and adamsite were present as well. As for the Southern Adriatic Sea, most of the dumped chemical weapons turned out to be American M47 aircraft bombs loaded with yperite. Since WWII two incidents involving fishermen have been registered and also a few involving tourists[29].

[28] Brankowitz, W. R., 1987. *Chemical Weapons Movement. History compilation*. Office of the Program Manager for Chemical Munitions. Aberdeen Proving Ground, MD, SAPEO-CDE-IS-87001.

[29] Plunkett G., 2003. *Chemical Warfare Agent Sea Dumping off Australia*. Australian Government – Department of Defence. ISBN 0 642 29587 28 pp. www.hydro.gov.au/n2m/dumping/cwa/chemical.pdf

4.1.6 Sea of Japan

In the Sea of Japan approximately 4,900 tons of CWAs, contained in 118,000 shells and 574,000 canisters, were dumped at sea [29] [30]. Even in these waters incidents involving fishermen have been reported. On one site in particular, 52 fishermen have been injured due to accidental recovery of CWAs probably loaded with yperite[30] (Fig. 4–2).

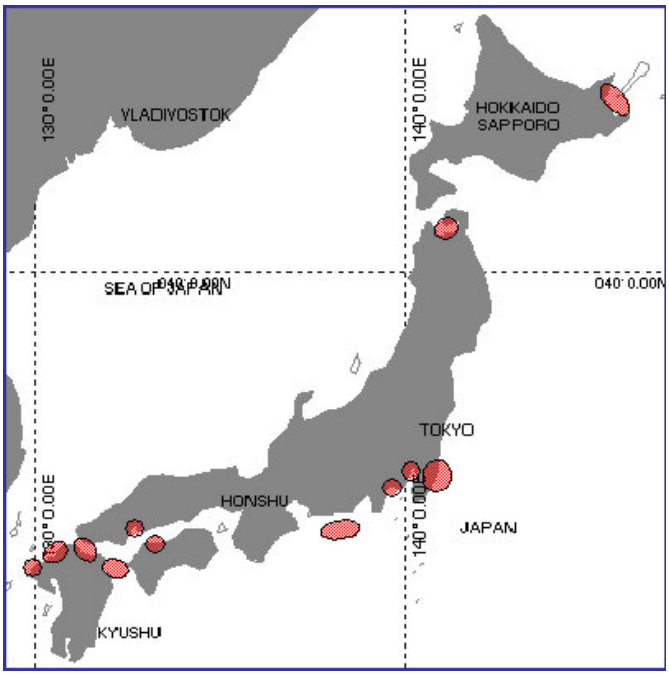

Fig. 4–16 Main dumping sites in the Sea of Japan[13]

[30] Kurata H, 1980. *Lessons learned from the destruction of chemical weapons of the Japanese Imperial forces. In: Chemical Weapons Destruction and Conversion*. Stockolm International Peace Research Institute (SIPRI). Taylor and Francis. London

5 The Italian dumping sites with particular reference to the Southern Adriatic Sea

In the same way as the other seas previously mentioned, the Mediterranean has also been theatre to dumping operations of obsolete war material. At the end of WWII the Southern Adriatic Sea represented the main dumping area in the basin for chemical weapons. The majority of the dumped materials came from the allies arsenals stocked in Southern Italy. As declared by the US Army[31], other Mediterranean chemical weapons dumping sites were located in front of Naples (Italy) and Saint Raphael (France) where the following ordnance were dumped respectively:

- unknown quantities of bombs loaded with phosgene, cyanogen chloride and hydrogen cyanide as well as 13,000 mustard mortar and artillery shells and 438 55-gallon drums filled with mustard gas;
- 1,700 lewisite bombs and 1,700 yperite bombs.

Several research activities have been carried out by authors with an aim to achieve a better knowledge on quantities and type of ordnance dumped in areas including the main Southern Adriatic Sea dumping areas. These activities include:

- interviews with fishermen;
- consultation of nautical charts and sailor notices, often reporting the official dumping areas as “unexploded ordnance area”;
- consultation of military and civilian archives

This research has allowed us to point out several dumping areas not only in the Southern Adriatic Sea but also in the territorial waters of foreign countries (Malta, Albania, Montenegro and Croatia). Nevertheless the identified areas probably do not represent the real number of dumping areas. With particular reference to the Southern Adriatic Sea, several interviews with fishermen highlighted both the greater extension of the dumping areas concerned and the existence of other dumping sites not officially reported (see par. 5.4 – CWs dumping sites).

[31] US Army Research, Development and Engineering Command, 2001. Off-shore disposal of chemical agents and weapons conducted by the United States. Historical database n° 26. 16 pp. http://www.dailypress.com/news/dp-02761sy0oct30,0,2199000.story

5.1 Historical framework of the Southern Adriatic Sea

Despite the fact blistering gases were only used on rare occasions during WWII (in China by the Japanese and during the invasion of Poland by the Germans) many countries, violating the Geneva Convention, continued to build up huge depots of chemical agents ready to use. In the Southern Adriatic Sea, in particular, the Allies had several munitions depots at their disposal mainly in the vicinity of Bari and Foggia. Several of them were stocked with chemical weapons, such as the General Depot n°5 in Bari, which had the task to restock the bombers of the 15th American Air Force.

By the end of WWII dumping activities in the Southern Adriatic Sea involved war surplus and ordnance recovered from naval wrecks, which had been sunk in Apulian ports and harbours, and resulted from clean-up activities as well as from the stores and production units mentioned above.

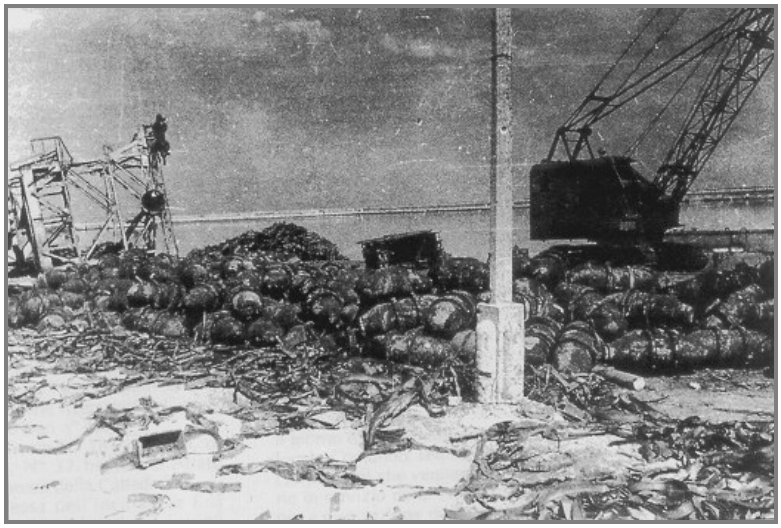

Fig. 5–1 Clean up operation of Bari harbour (source: State Archives in Bari)

The seafloor of the Southern Adriatic ports were obstructed by a huge quantity of ordnance, dispersed following aerial raids and accidental dumping which occurred during loading/unloading operations.

The Bari raid, carried out on the 2nd of December 1943 by 105 Ju-88 bombers of the Luftwaffe fleet, represents one of the main causes which led to the presence of chemical weapons on the seafloor of the Southern Adriatic Sea. The bombing caused the explosion of the U.S.S. JOHN HARVEY in Bari' harbour, which was

then dumped together with another 16 ships (5 American, 4 English, 2 Norwegian, 3 Italian and two Polish) [32] [33].

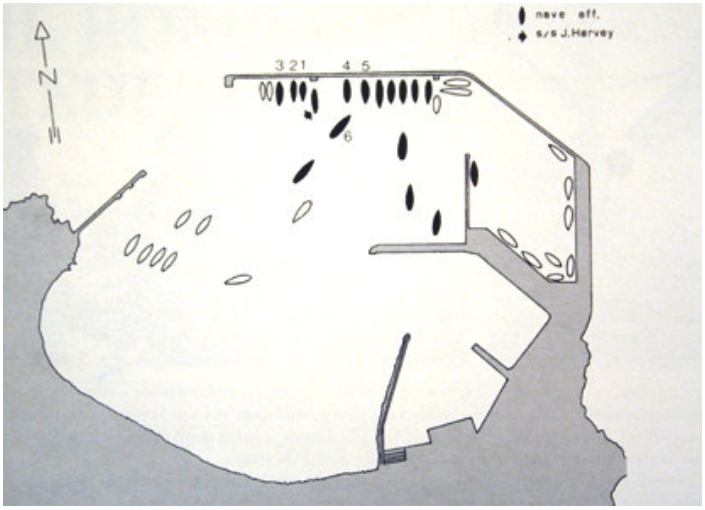

Fig. 5–2 Position of wrecks in Bari harbour after the German raid of the 2nd of December 1943 (source: State Archives in Bari)

Fig.5–3 The day after the German raid 2 December 1943 (source: documentary "Combat Film" - Istituto Luce)

[32] Infield Glenn B., 2003. *Disastro a Bari*. Adda Ed.. Bari

[33] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 641 Fascicolo 3229. "Planimetria generale del Porto di Bari in cui vengono indicate la posizione, il tipo e la quantità degli ordigni rinvenuti e rimossi".*

The American Liberty was secretly loaded with at least 15,000 aircraft bombs[34] M47A1 and M70 filled with yperite, belonging to depot n°5 of Bari and ready to be used in case of a similar enemy attack [32]. This great naval disaster had serious consequences mainly on the population of Bari as more than one thousand victims including soldiers and civilians were registered. Some of the victims were affected by yperite others by the fatal mixture of yperite and naphtha that contaminated the waters of the harbour. A recent official note by the American Government states that on these occasion there were 53 victims of yperite, while the total number of injured people was 534 [35]. It is probable that the secrecy of the operations may have caused a significant delay in the employment of the correct assistant for the injured [32] [36].

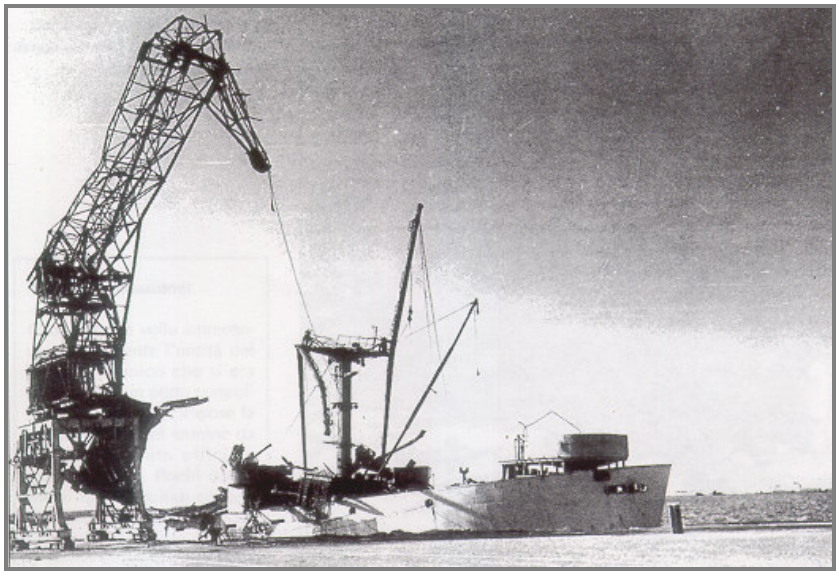

Fig. 5–4 Salvaging of USS JOHN HARVEY in the harbour of Bari at the end of WWII (Source: *Associazione Marinai d'Italia* (Italian Sailors Association) of Bari)

Clean up operations were carried out in the port of Bari, as well as in other Italian bombed harbours, where wrecks and munitions were recovered and subsequently dumped offshore. The main apulian ports involved were Manfredonia, Margherita di Savoia, Barletta, Trani, Molfetta, Bari, Brindisi and Taranto.

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[34] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 641 Fascicolo 3229. Dicembre 1952 – "Rapporto riassuntivo del Nucleo Sminamento Porti Puglie dei lavori di bonifica nel porto di Bari relativo al Piroscafo n°3 (USS John Harvey)"*

[35] Mitretek Systems, 2004a. Ocean dumping of chemical weapons. www.mitretek.org/home.nsf/homelandsecurity/OceanDumpChemWeap

[36] Ferrari P., Leuzzi V.A., 1994. *Il bombardamento tedesco su Bari*. Italia Contemporanea, **196**: 4-12.

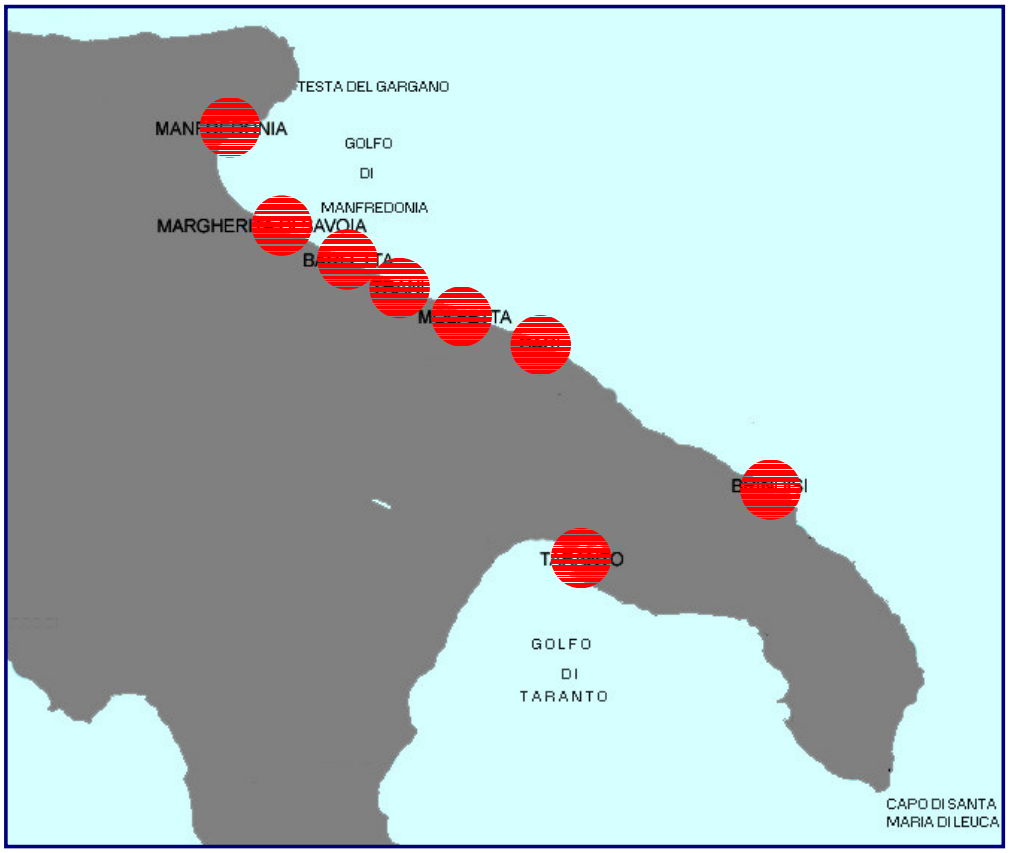

Fig. 5–5 Map representing the location of the main ports involved in clean up operations

The huge quantity of CWAs which is at present located on the seafloor of the Southern Adriatic Sea is due not only to the remediation activities mentioned above, but also to the dumping of war materials from English and American arsenals located along the apulian coast. Historians usually report that in Puglia "...preparations were made for a chemical war which was never declared but at the same time very well organised by the army..." [37].

As a matter of fact, both the Germans and the Allies dumped chemical weapons (drums of phosgene and yperite and various chemical ordnance) which were transported off-shore by barges and ships. As reported by eyewitnesses, since the beginning of 1944 the ports of Molfetta and Manfredonia were chosen as preferential sites for loading ships with chemical and conventional munitions which were intended to be dumped later. A report made by the Italian *Carabinieri* in March 1944 describes a serious incident which occurred while

[37] Leuzzi V.A., 1996. *Aggressivi chimici e guerra. La contaminazione in Puglia 1943-1996.* Italia Contemporanea, **206**: 149-156.

transporting some hazardous war material that were meant to be dumped on a big barge off the coast at the port of Manfredonia. On this occasion five soldiers (four English and one Italian) lost their lives and several others were injured [38].

Interviews with fishermen confirm only the partial responsibility of port' remediation activities for the high quantity of dumped ordnance in the Southern Adriatic Sea. By 1946, one year prior to the beginning of the clean up operations, a significant number of fishermen has been injured by the accidental recovery of chemical weapons trapped in their trawling nets[39]. In a letter dated 26 August 1960 addressed to the Molfetta Mayor, a worker employed in the English depots located along the apulian coast refers that he was involved mainly in the dumping at sea of bombs which had not been utilised. The ordnance was first loaded on ships in the Molfetta harbour and later dumped at sea often in proximity to the port [40].

Through the testimony of local people it has been possible to discover the presence of an area which was managed by Americans in the southern part of Manfredonia where munitions depots were located. An interview dated 20 September 2005 (record available at ICRAM) with Giovanni Ricucci, employed during the war in these depots, confirms that millions of bombs (some loaded with yperite or white phosphorus) were stocked there. At the end of the war the most dangerous ones were dumped at sea using fishing vessels.

Other sources have identified the presence of the chemical plant "Dott. Saronio" near Foggia. Officially known as a beer-producing factory, in 1941 it was able to produce around 200 tons of yperite and 100 tons of diphosgene per month. The factory was also using, as raw material, chlorine produced in the nearby cellulose factory [41]. On the 28 September 1943 the Germans in retreat destroyed the chemical plant [42] and, as reported in a document by the *Nucleo Sminamento Porti Puglie* (Apulian Ports Demilitarisation Centre) [43], the

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[38] State Archives in Bari. Prefettura, Gabinetto, III vers. B. 218

[39] Mastrorilli A., 1958. *Esiti a distanza di lesioni di vescicatori - revisione clinico-statistica su 102 casi.* Giornale di Medicina Militare, **4**: 352-361.

[40] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 93*

[41] Rochat G. 1996. *L'impiego dei gas nella guerra d'Etiopia 1935-1936*. In Del Boca A., 1996. *I gas di Mussolini*, Editori Riuniti

[42] Leuzzi V.A., 1995. *La fabbrica della morte dei tedeschi a Foggia*. Newspaper *Gazzetta del Mezzogiorno*, May 15th 1995.

[43] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 641*

remaining fuses and chemical weapons were recovered and transported to Bari harbour in order to be dumped at sea.

Fig. 5–6 The Chemical Plant "Dott. Saronio" located near Foggia produced Yperite and diphosgene during WWII (source: ICRAM)

The remediation activities of the Apulian ports started only some years after, in 1947, due to the risks and difficulties related to the huge amount of chemical weapons which were present in the area. In April 1946 the *Ministero dei Lavori Pubblici/Direzione Generale Opere Marittime* (Italian Ministry for Public Works) decided to start the clean up operations in the ports and to proceed with the detection of hulls, wrecks and other material. In 1947 the Service *Porti Demanio e Pesca* of the *Ministero della Marina Mercantile* (Italian Merchant Marine Ministry) issued a directive addressed to port authorities, which clearly indicated the main rules for a safe removal of ships carrying explosive or chemical ordnance. According to this directive, chemical warfare agents were supposed to be dumped at sea in areas having a minimum depth of 460 meters, at a distance of at least 20 nautical miles from the coast and 10 nautical miles from the nearest traffic route[44].

[44] State Archives of Italian Navy in Rome. *Archivio Santoni, cartella 30, fascicoli 15-16*.

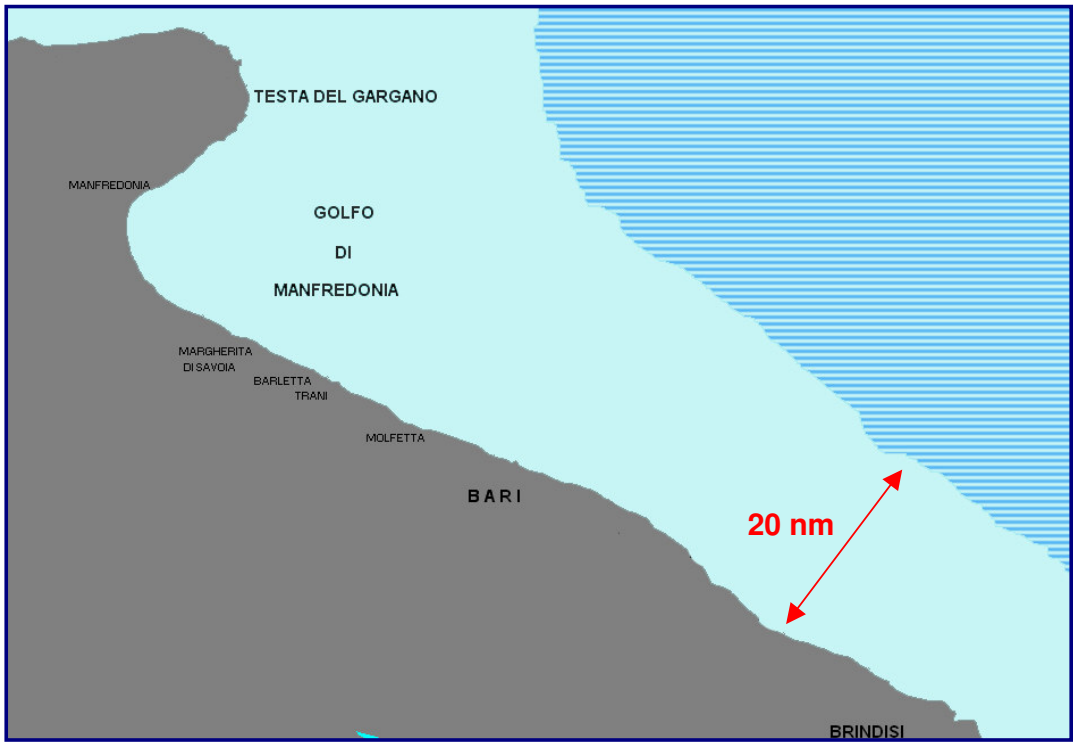


Fig. 5–7 Map showing the areas of the Southern Adriatic Sea where it was possible to dump chemical weapons following the Italian Merchant Marine Ministry directive (source: ICRAM)

The Apulian Ports Demilitarisation Centre was specifically created to deal with the clearance of chemical weapons from ports. Remediation activities were carried out between 1947 and 1953, mainly in the Port of Bari and, for a limited period of time (a few weeks), in other Apulian ports affected by the presence of dumped chemical ordnance. This was the case in Molfetta harbour, which was partially cleaned up between January and March 1948.

Many of the explosive devices recovered were found to be loaded with chemical agents[45] [46].

In Bari harbour professional divers carried out salvaging operations and chemical weapons were kept either in underwater metal cylinders or in a specific depot. Personnel working at the deposit were provided with safety equipment and protective clothing against yperite contamination.

[45] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 282 Fascicolo 1341*. "*Relazione del Nucleo Sminamento Porti Puglie di fine lavori delle attività di sminamento e bonifica del porto di Molfetta".*

[46] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 282 Fascicolo 1341*. "*Planimetria generale del Porto di Molfetta in cui vengono indicate la posizione, il tipo e la quantità degli ordigni rinvenuti e rimossi".*

Fig. 5–8 Chemical weapons M47A1 transported to Bari harbour by Apulian Ports Demilitarisation Centre operators (source: State Archives in Bari)

Fig. 5–9 Chemical drum transported to Bari harbour by Apulian Ports Demilitarisation Centre operators (source: State Archives in Bari)

Fig. 5–10 Drum used to contain the protection devices against Yperite contamination (source: State Archives in Bari)

However, due to the highly volatility of yperite, incidents were rather frequent, especially during the hot season[47].

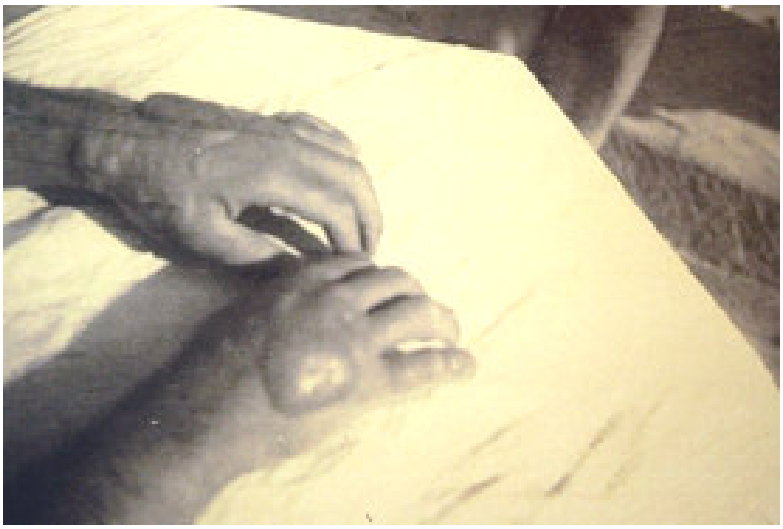


Fig. 5–11 Hands of a diver injured by leakage of yperite during clean up operations inBari harbour (source: State Archives in Bari)

Salvaging operations concerned not only the USS JOHN HARVEY but also the U.S.S. CHARLES HENDERSON, which sank on 9 April 1945 in Bari harbour, following an apparently accidental explosion. At the moment of the accident, the ship was carrying around 8,000 bombs, mainly loaded with yperite. The operators of the Apulian Ports Demilitarisation Centre defined the Henderson's load as "too dangerous to be kept on land or to be handled as necessary for demilitarisation aims"[48].

[47] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 641 Fascicolo 3229*. "*Rapporto settimanale di attività (21-30 giugno 1949) del Nucleo Sminamento Porti Puglie dei lavori di bonifica del porto di Bari*"

[48] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 677*

Fig. 5–12 Recovery of aerial bombs from the hull of U.S.S. CHARLES HENDERSON (source: State Archives in Bari)

Fig. 5–13 Stockage on the quay of Bari harbour of aerial bombs, ready for dumping and recovered from the hull of U.S.S. CHARLES HENDERSON (source: State Archives in Bari)

Part of the war material dumped on the seafloor of the Southern Adriatic Sea derives from the recovery activities of iron materials carried out by the ordnance unpacking firms. These include the very well known "*Polverifici Giovanni Stacchini*", which in 1950 signed a contract with the Bari Port Authority of for the recovery of dumped ordnance[49]. In order to carry out this operation, the Stacchini firm utilised hundreds of fishing vessels to recover dumped ordnance using trawling nets. Besides conventional weapons, chemical weapons were also accidentally collected. Even though chemical ordnance was promptly released back into the water as soon as it was recognised, a significant number of chemical weapons was transported by mistake on land and finally abandoned along the coast at a few meters of depth. After the bankruptcy of the firm, clean up operations of the ordnance left in the shallow waters of *Torre Gavetone*, located between Molfetta and Giovinazzo, started in 1960 and ended only in 2002. An official document provided by the Molfetta Cost Guard confirms that, during the remediation activities carried out in the area, 926,000 unexploded

[49] Archives of Molfetta Harbour-office Commander. *Contratto stipulato tra Capitaneria di Porto di Bari e la Società "Polverifici Giovanni Stacchini"*

ordnance were recovered and destroyed and, among these, 1,157 were chemical weapons and 300 were filled with white phosphorus[50].

Fig. 5–14 Construction between Molfetta and Giovinazzo where the Stacchini firm was located. On the seafloor near the shoreline the underwater operators of the Italian Navy have recovered hundreds of thousands of ordnance (source: ICRAM)

Similar activities were carried out by the ULMER firm, located south of Manfredonia, which had the task of recovering the huge quantity of war material abandoned by the English and the Americans.

Dumping operations at sea were performed by at least three ships: M/c LETE, M/S 75 and M.A.S. 547, equipped with special devices able to dump war material (slides, opening bottom). With regards to dumping sites, the monthly reports usually refer to "limited areas of the seafloor". However only a few reports clearly indicate 2 dumping sites located respectively 40 nm off Bari at 1000m of depth for chemical weapons and 15 nm off Bari at 400m of depth for both conventional and chemical weapons[51]. The latter most probably corresponds to the area which was indicated by fishermen and located in front of *Mola di Bari* (see map of the Southern Adriatic Sea dumping areas, Annex II).

[50] Molfetta Cost Guard Archive. *Relazione Rinvenimento Ordigni Località Torre Gavetone* (Molfetta)

[51] State Archives in Bari. *Genio Civile – Opere Marittime – Busta 641 Fascicolo 3229*. "*Rapporto settimanale di attività (8-14 ottobre 1947) del Nucleo Sminamento Porti di Bari*".

The correspondence seems to be confirmed also by the information gained from the logbooks of the afore mentioned ships on some their travels[52] [53].

Fig. 5–15 Aerial bombs model *M47 A1* loaded with yperite, recovered from Bari harbour seafloor and ready for dumping in Southern Adriatic Sea (source: State Archives in Bari)

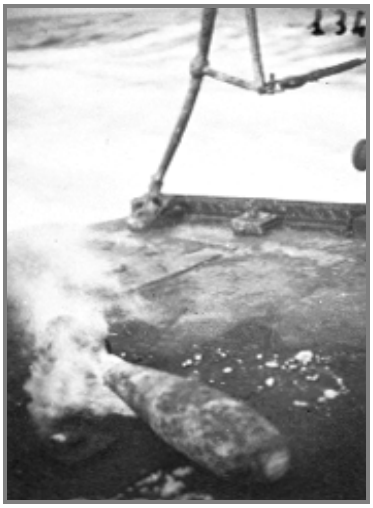


Fig. 5–16 Artillery bomb on the stern of a military vessel during the dumping operation (source: State Archives in Bari)

A report of the Apulian Ports Demilitarisation Centre, which describes the chemical weapons recovered from Bari harbour between 1949 and 1953, clearly indicates, among others, 15,551 aerial bombs and 2,533 boxes with ammunition both loaded with yperite.

Moreover, another report of the Apulian Ports Demilitarisation Centre states that on the 5th of June 1953 1,622 chemical bombs loaded with yperite were dumped into the "limited areas" by the military barge "G.A. 61". Then, the total quantity of chemical weapons officially dumped in Southern Adriatic sea is 19,706.

Although dumping operations entrusted to the Italian Navy have probably been carried out within the so-called "limited areas", for economic reasons several operations were performed by fishing vessels which usually dumped the munitions in different areas closer to the coast in order to limit fuel consumption.

[52] State Archives of Italian Navy in Rome. *Navi e imbarcazioni, Fascicolo M-32.* "*Rapporti di Navigazione del S.T. di Vascello Orazio Luigi Marzi, Comandante della M/s75 durante le operazioni di affondamento di bombe caricate con Iprite"*

[53] State Archives of Italian Navy in Rome. *Navi e imbarcazioni, Fascicolo L-3. "Rapporto di Navigazione del Comandante della M/c "Lete" durante le operazioni di affondamento di bombe ed esplosivi recuperati nel porto di Bari".*

Fig. 5–17 Aerial bombs model *M47 A1* loaded with yperite, transported by a local fish vessel from Bari harbour to an undefined sea dumping area in Southern Adriatic Sea (source: State Archives in Bari)

As a matter of fact, all these events have led to the present uncertainty as regards both position and distribution of dumping areas in the Southern Adriatic Sea. As better specified in par. 5.2 the dispersion of munitions at sea was also enhanced during the following decades by fishing activities.

It is of relevance to stress that all the information gained by consulting the military archives is incomplete, in some cases vague and that the given data is most probably underestimated. Considering the 926,000 unexploded ordnance recovered at sea near *Torre Gavetone* (as mentioned above) it is presumed that the real number could be at least ten times this figure (19,706 chemical weapons).

5.2 Interviews with fishermen

Interviews with fishermen have represented a very important source of information considering that for 6 decades fishermen have been victims of numerous incidents due to the accidental recovery of chemical weapons in their trawling nets. A large number of unreported incidents have been discovered by this present study and must be added to the official 223 cases of fishermen hospitalised (see page 11). In many cases fishermen have preferred not to report the event to the authorities, especially when accidental recoveries

occurred in areas forbidden to fishing activities. Moreover, by notifying the event, they would have been obliged to wait for the intervention of the Italian Navy's demilitarisation team, with a consequent loss of working days.

Consequently fishermen preferred to abandon the bombs at sea, preferentially in areas where they don't fish (often near the ports), thus contributing to a further dispersion of the bombs on the seafloor.

A study carried out by the University of Bari[54] reveals several cases of accidental exposure to chemical agents, mainly yperite, contained in the recovered bombs.

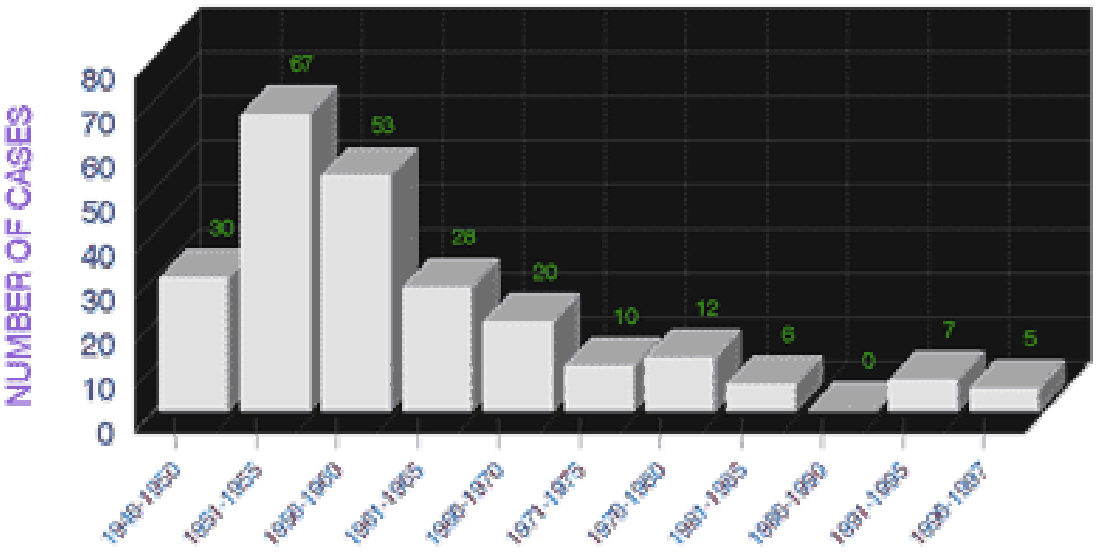


Fig. 5–18 Distribution of injuries of fishermen during the period 1946 – 1997 in the Southern Adriatic Sea. Source: Miretek Systems, 2005f [55]

[54] Assennato G., Ambrosi F., Sivo D., 1997. *Possibili effetti a lungo termine sull'apparato respiratorio della esposizione ad iprite tra pescatori*. La Medicina del Lavoro, **88** n°2.

[55] Miretek Systems, 2005f. *Continuing Exposure to Mustard in the Adriatic*. www.mitretek.org/ContinuingExposureToMustardInTheAdriatic.htm

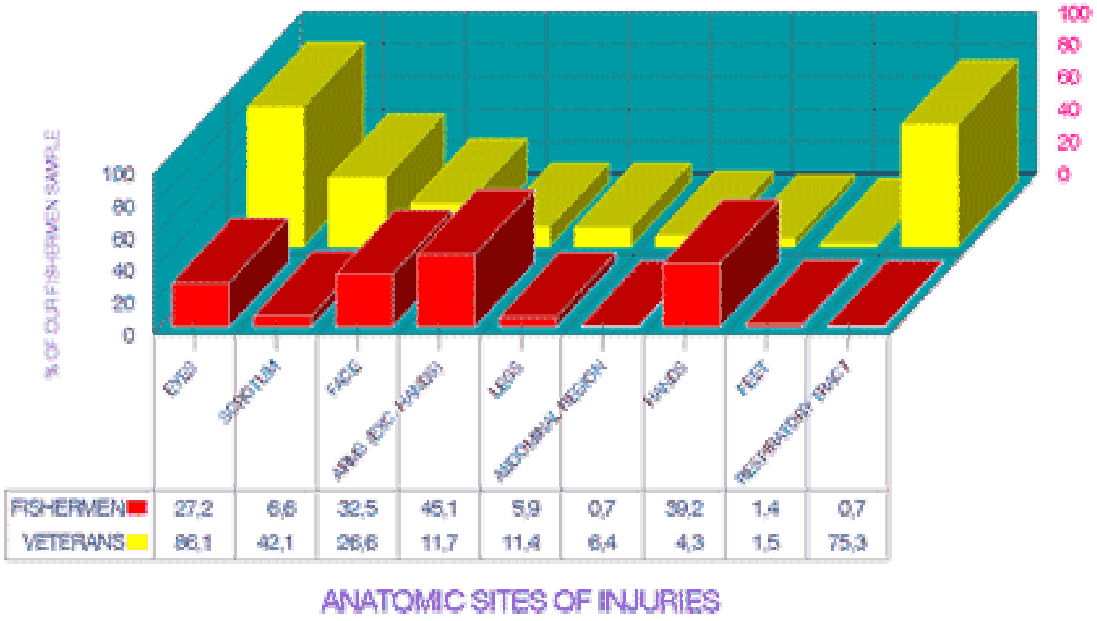


Fig. 5–19 Frequency distributions of injuries. Comparison between fishermen in the Southern Adriatic Sea (135 cases) and American veterans of WWI (7,000 cases). Source: Miretek Systems, 2005f

Both acute (skin and eyes blisters) and long term effects to the respiratory apparatus were registered.

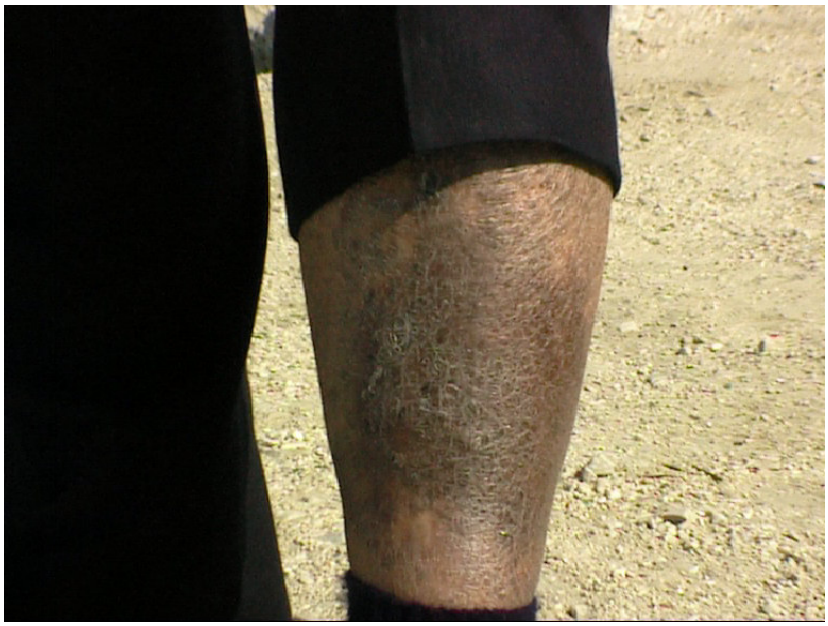


Fig. 5–20 The legs of a fisherman with skin damaged after the accidental recovery of a chemical weapon

In order to collect data in a uniform manner, the fishermen were provided with a specific questionnaire (see Annex III) asking for detailed information regarding events of accidental recovery of chemical weapons at sea. Eightfour fishermen working in the Southern Adriatic Sea (ports of Manfredonia, Barletta, Trani, Molfetta, Bari, Mola di Bari, Monopoli, Brindisi, Otranto) were interviewed. In several cases the incidents reported referred to contamination by yperite. Fishermen refer that as soon as the bombs were taken on board they were able to identify a strong odour of garlic, typical of yperite. Direct contact with this agent, either leaking from the bombs or present on the trawling nets, after having fished on the seafloor contaminated by yperite, affected the fishermen causing severe blistering effects. Fishermen report that yperite was mostly present in its solid phase although in some cases, in particular in the area located 20 nautical miles off Mola di Bari (see map of Southern Adriatic Sea, Annex II) the product appeared in its liquid phase maintaining both the odour and its effects unaltered. A bibliographic research allowed us to discover that several countries, particularly in areas with a cold climate, used to lower the solidification temperature of yperite by mixing it with arsenic compounds in order to facilitate its use also during the winter season.

Fig. 5–21 Rusted aerial bomb accidentally caught by fishermen in the Southern Adriatic Sea (source: ICRAM)

In other cases, particularly near Manfredonia harbour, fishermen report major contamination by white phosphorous, which burns as soon as it comes in contact with the atmosphere.

Fig. 5–22 white phosphorous in water

Fig. 5–23 The same piece of white phosphorous starts to burn as soon as it comes in contact with the atmosphere

The interviews have enabled us to identify new dumping areas. According to the type of ordnance accidentally recovered the same areas have been further divided into 3 zones: chemical weapons loaded with yperite (4 areas), white phosphorous (1 area) and conventional weapons (6 areas) (see map of Southern Adriatic Sea, AnnexII). Several areas identified by fishermen have also been confirmed by coast guard reports and detailed nautical charts. Vice versa, an area located 20 nautical miles off Mola di Bari, indicated by the archival research as one of the main dumping areas of the Italian Navy has been confirmed by fishermen interviews (see also par. 5.1).

With the aim of providing fishermen with solid support in case of accidental recovery of chemical weapons (Appendix I), ICRAM, in collaboration with CETLI, has prepared a handbook illustrating the main precautionary measures to take as well as suggestions regarding procedure and the main measures to be taken regarding first aid.

5.3 Other sources

As already mentioned before, the localisation of the main dumping areas has been achieved also through consultation of nautical charts, sailor notices and documents recovered from both military and civilian archives.

The consultation of nautical charts and sailor notices allowed us to identify 24 dumping areas often marked as “unexploded ordnance area”. Although the majority of these areas is located in the Adriatic Sea, other similar areas have been found near Malta and in the Gulf of Naples. The latter probably

corresponds to the area declared by the US Army where chemical weapons were dumped after WWII (see page 10).

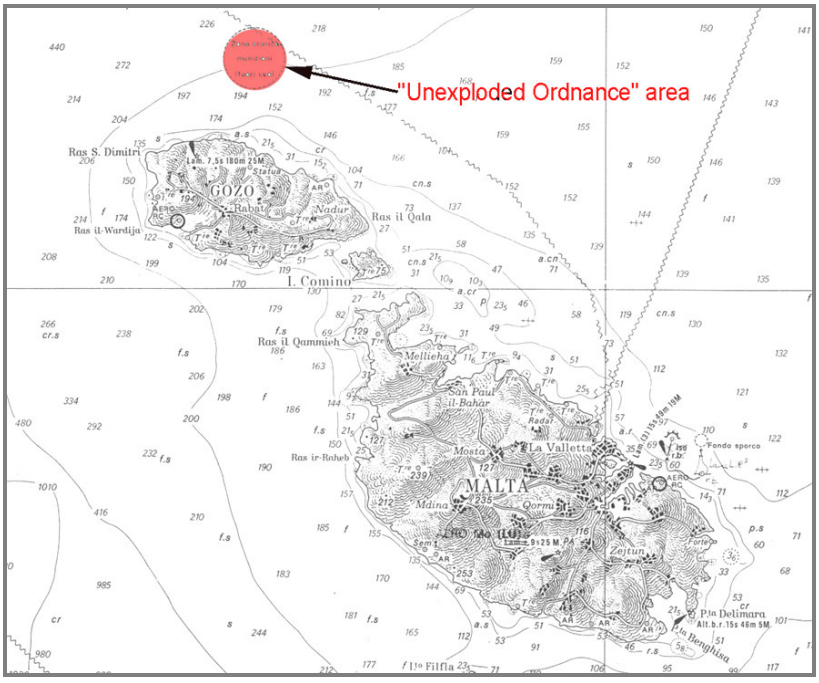


Fig. 5–24 "Unexploded ordnance area" north of Malta

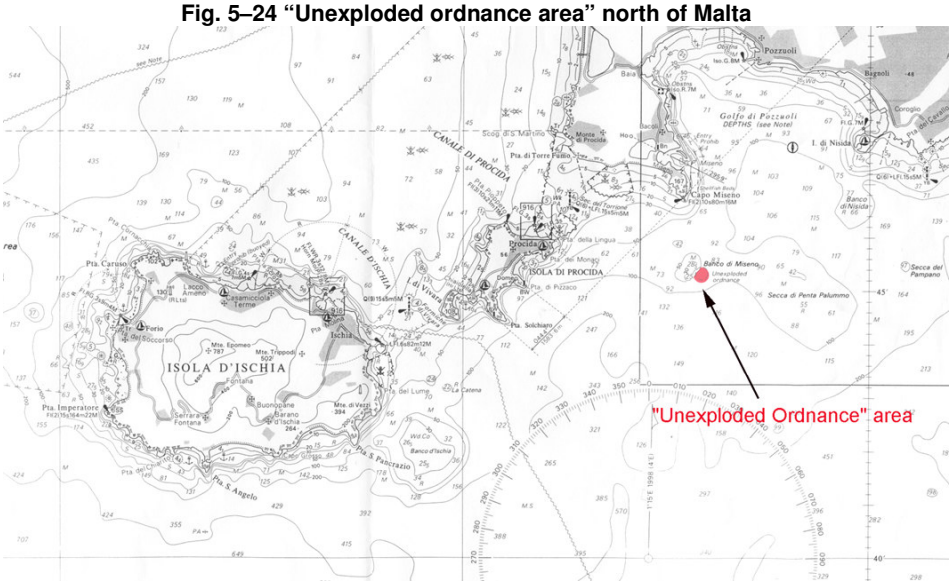


Fig. 5–25 "Unexploded ordnance area" in the Gulf of Naples

Sailor notices allowed us to add several areas not marked on nautical charts. In fact, as confirmed by the Italian Hydrographic Institute, the indication of dumping areas on the official charts is usually deleted after some decades; thus it is only possible to trace the areas using sailor notices. In this way, dumping

areas were identified in the Ligurian Sea, around Sardinia and off Venice, where a ship loaded with war surplus materials was dumped (see map of Italian sea dumping areas, Annex I).

Both military and civilian archives have represented other important sources of information. The most useful archives are reported below.

- State Archives in Bari for data related to the clean up operations of Apulian harbours and to the dumping of munitions deriving from the allies' arsenals;

- State Archives of the Italian Navy in Rome for data related to the dumping operations carried out by ships of the Italian Navy.

Two official dumping areas have been located: the first one 40 nautical miles off Bari at 1000 m of depth and the second 20 nautical miles off Mola di Bari at 300-500 m of depth. The main results of this research project are reported in paragraph 5.1 on historical framework.

Finally, the Italian coast guard archives completed our set of information for the Southern Adriatic Sea. These archives, for instance, were fundamental for the identification of the area near Molfetta (Torre Gavetone) wherethe Italian Navy recovered and destroyed 926,000 unexploded ordnance, which included 1,157 chemical weapons (paragraph 5.1). At present the area is interdicted as other war material is still lying on the seafloor.

The Italian Navy in 2005 carried out another recovery operation of chemical weapons at sea in Molfalcone harbour (Northern Adriatic Sea). In this case about 150 artillery munitions dumped after the WWI were collected. All the munitions loaded with mustard gas were transported to Civitavecchia for demilitarisation by the CETLI. The following pictures represent different phases of this operation.

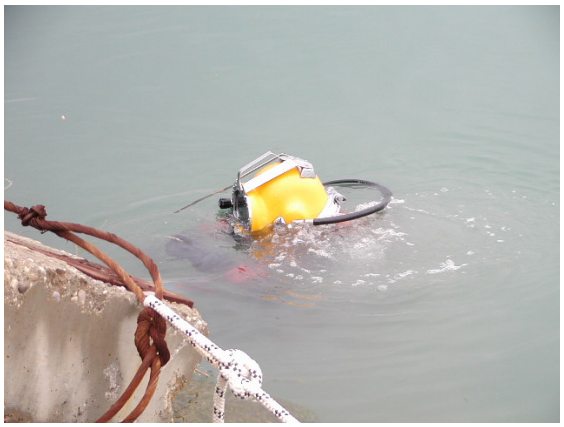


Fig. 5–26 Italian Navy underwater operator equipped with a protective suit during the recovery operation of CWs in MoInfalcone harbour (source: CETLI)

Fig. 5–27 CWs in MoInfalcone harbour slinged for their recovery (source: CETLI)

Fig. 5–28 Mechanical recovery of CWs (source: CETLI)

Fig. 5–29 Temporary storage of CWs (source: CETLI)

Fig. 5–30 Temporary storage of CWs (source: CETLI)

Fig. 5–31 Some of the CWs recovered in Monfalcone harbour showing the leakage of a black oily substance, where the yperite was the main substance (source: CETLI)

5.4 CW Dumping sites

The historical research together with the interviews with fishery stakeholders, the nautical charts and the sailor notices allowed us to identify 55 dumping areas along the Italian coast. Their main characteristics are reported in table 5-1 below whereas their distribution and extension are illustrated in maps reported in Annexes I and II.

Table 5–1. The main Italian sea dumping areas

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Northern Adriatic Sea	Coordinates of centre: ϕ 44°53'.400N; λ 013°24'.900E Radius 4,35 nm	41	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 39/1991
Northern Adriatic Sea	Coordinates of centre: ϕ 45°06'.150N; λ 012°59'.100 Radius 0,4 nm	31	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 38/1992
Northern Adriatic Sea	Coordinates of vertexes: A ϕ 45°39'.190N; λ 013°44'.950 E B ϕ 45°39'.520N; λ 013°44'.950 E C ϕ 45°39'.520N; λ 013°44'.450 E D ϕ 45°39'.270N; λ 013°44'.450 E E ϕ 45°39'.190N; λ 013°44'.650 E	20	Polygon near Trieste marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 239/1994
Southern Adriatic Sea	Coordinates of centre: ϕ 41°56'.350; λ 016°16'.600 E Radius 1,4 nm	50	Circular area in front of Gargano marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 32/2000
Central Adriatic Sea	Coordinates of centre: ϕ 42°08'.200N; λ 018°31'.100 E Radius 2,5 nm	200	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 6011/1990
Sicilian Channel	Coordinates of centre: ϕ 36°07'.400N; λ 014°15'.430 E Radius 1,5 nm	200	Circular area north of Malta marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 917/1986

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Central Adriatic Sea	Coordinates of centre: ϕ 42°08'.300N; λ 018°32'.400 E Radius 3 nm	230	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 6012/1997
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°30'.400N; λ 018°59'.830 E B ϕ 40°30'.400N; λ 018°59'.960 E C ϕ 40°14'.980N; λ 019°09'.970 E D ϕ 40°15'.400N; λ 019°25'.780 E E ϕ 41°30'.700N; λ 019°31'180 E	From 0 to 500	Polygon in Albania marked on nautical charts as "mined field"	NAUTICAL CHART n° 6012/1997
Southern Adriatic Sea	Coordinates of centre: ϕ 41°33'.580N; λ 016°16'.500 E Radius 1,5 nm	300	Circular area in front of Bari marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 31/1991
Southern Adriatic Sea	Coordinates of centre: ϕ 41°37'.000N; λ 015°54'.750 E Radius 0,5 nm	10	Circular area in front of Manfredonia marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 199/1987
Northern Tyrrhenian Sea	Coordinates of centre: ϕ 42°54'.670N; λ 005°53'.670 E Radius 0,5 nm	200	Circular area in front of France marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 908/2001
Southern Adriatic Sea	Coordinates of centre: ϕ 41°41'.400N; λ 017°47'.670 E Radius 5 nm	1.200	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 921/2001
Southern Adriatic Sea	Coordinates of centre: ϕ 42°00'.000N; λ 017°00'.000 E Radius 3 nm	550	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 921/2001

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Southern Adriatic Sea	Coordinates of centre: ϕ 41°55'.800N; λ 017°25'.500 E Radius 9 nm	1.020	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 921/2001
Southern Adriatic Sea	Coordinates of centre: ϕ 41°18'.170N; λ 018°28'.170 E Radius 5 nm	1.000	Circular area in the middle of Southern Adriatic sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 921/2001
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°28'.000N; λ 018°19'.00 E B ϕ 41°28'.000N; λ 018°31'.000E C ϕ 41°45'.000N; λ 018°20'.000E D ϕ 41°45'.000N; λ 018°51'.000E	1.100	Rectangular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 921/2001
Southern Adriatic Sea	Coordinates of centre: ϕ 39°49'.000; λ 019°00'.000 E Radius 1 nm	-	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 920/2000
Southern Adriatic Sea	Coordinates of centre: ϕ 40°41'.000N; λ 018°29'.500 E Radius 0,5 nm	-	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHARTn° 920/2000
Southern Adriatic Sea	Coordinates of centre: ϕ 40°49'.000N; λ 018°51'.670 E Radius 4,6 nm	-	Circular area in the middle of Southern Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 920/2000
Central Adriatic Sea	Coordinates of centre: ϕ 43°08'.330N; λ 015°26'.000 E Radius 2,5 nm	-	Circular area in the middle of Central Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 922/2005

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Central Adriatic Sea	Coordinates of centre: ϕ 43°58'.850N; λ 014°46'.610 E Radius 1 nm	-	Circular area in the middle of Central Adriatic Sea marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 923/2004
Northern Adriatic Sea	Coordinates of centre: ϕ 44°25'.240N; λ 014°21'.800 E Radius 4 nm	-	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 923/2004
Northern Adriatic Sea	Coordinates of centre: ϕ 44°51'.400N; λ 013°44'.670 E Radius 2 nm	40	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 924/2004
Northern Adriatic Sea	Coordinates of centre: ϕ 44°20'.000N; λ 014°21'.670 E Radius 4 nm	50	Circular area marked on nautical charts as "unexploded ordnance dumping area"	NAUTICAL CHART n° 924/2004
Southern Adriatic Sea	Coordinates: ϕ 40°59'.900N; λ 017°15'.600E	40	Shipwreck probably loaded with conventional weapons	Interviews with the Monopoli coast guard and fishermen
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°11'.890N; λ 016°38'.220 E B ϕ 41°11'.810N; λ 016°38'.560 E C ϕ 41°11'.660N; λ 016°38'.520 E D ϕ 41°11'.690N; λ 016°38'.180 E	0-10	Rectangular area wherethe underwater operators of the Italian Navy recovered and destroyed 926,000 unexploded ordnance, among these 1,157 were chemical weapons and 300 were filled with white phosphorus. At the moment the area is forbidden to public use as well as to fishing activities.	Coast guard of Molfetta

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Central Adriatic Sea	Coordinates of vertexes: A ϕ 42°13'.670N; λ 015°44'.510 E B ϕ 42°13'.670N; λ 015°45'.200 E C ϕ 42°13'.270N; λ 015°45'.200 E D ϕ 42°13'.270N; λ 015°44'.510 E	0-50	Rectangular area around Pianosa island where ICRAM underwater operators have observed several conventional aerial bombs	ICRAM
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 40°05'.590N; λ 018°30'.180E B ϕ 40°05'.590N; λ 018°30'.280E C ϕ 40°05'.440N; λ 018°30'.140E D ϕ 40°05'.440N; λ 018°30'.280E	30	Rectangular area around the islet of S. Emiliano, near Otranto Cape, where the presence of conventional weapons on the seafloor have been recorded	Coast guard of Otranto
Southern Adriatic Sea	Coordinates: ϕ 41°32'.400N; λ 017°22'.530E	1.000	Area located about 34 nautical miles off Bari with a direction of 45° where the military ship MS75 sank tens of chemical weapons loaded with yperite	Italian Navy Archives – daily register of military ship MS75
Southern Adriatic Sea	Coordinates: ϕ 41°31'.090N; λ 017°24'.160E	1.000	Area located about 34 nautical miles off Bari with a direction of 48° where the military ship MS75 sank tens of chemical weapons loaded with yperite	Italian Navy Archives – daily register of military ship MS75
Southern Adriatic Sea	Coordinates: ϕ 41°18'.000N; λ 017°06'.000E	400	Area located about 20 nautical miles off Bari where the military ship MC LETE sank tens of chemical weapons loaded with yperite	Italian Navy Archives – daily register of military ship MC LETE
Southern Adriatic Sea	Coordinates: ϕ 41°21'.830N; λ 017°12'.000E	400	Area located about 20 nautical miles off Bari where the military ship MC LETE sank tens of chemical weapons loaded with yperite	Italian Navy Archives – daily register of military ship MC LETE
Ionian Sea	Coordinates: ϕ 40°19'.450N; λ 017°12'.190E	300	Area located about 5 nautical miles off Taranto where the Italian Navy sank nearly 800 conventional and chemical weapons coming from the clean up operations of Taranto harbour	Italian Navy Archives

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°48'.800N; λ 016°54'.783E B ϕ 41°43'.866N; λ 016°55'.833E C ϕ 41°43'.550N; λ 016°53'.166E D ϕ 41°48'.483N; λ 016°52'.116E	230	102 targets located on the seafloor during a survey carried out in 1999 (A.C.A.B. project) using side scan sonar, sub bottom profiler and magnetometer. 9 aerial and 2 artillery bombs loaded with yperite have been inspected usin a ROV,	ICRAM
Northern Adriatic Sea	Coordinates: ϕ 45°17'.250N; λ 012°35'.050E	14,5	Ship loaded with conventional weapons located near Venice lagoon	MEDITERRANEAN PILOT BOOK VOL. 1C 1982
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°12'.850N; λ 016°35'.010E B ϕ 41°12'.950N; λ 016°35'.310E C ϕ 41°12'.850N; λ 016°35'.350E D ϕ 41°12'.750N; λ 016°35'.060E	5	Area immediately outside Molfetta harbour where in 1999, ICRAM underwater operators observed tens of weapons on the seafloor	ICRAM
Northern Adriatic Sea	Coordinates: ϕ 45°26'.680N; λ 012°34'.940E Radius 0,5 nm	30	Circular area near Trieste. Presence on the seafloor of conventional weapons	MEDITERRANEAN PILOT BOOK VOL. 1C 1982
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°22'.510N; λ 016°14'.270E B ϕ 41°21'.060N; λ 016°15'.600E C ϕ 41°20'.610N; λ 016°15'.290E D ϕ 41°22'.040N; λ 016°13'.430E	10	Rectangular area between Ofanto river and Barletta. Presence on the seafloor of conventional weapons	MEDITERRANEAN PILOT BOOK VOL. 1C 1982
Northern Tyrrhenian Sea	Coordinates: ϕ 44°02'.570N; λ 009°41'.810E Radius 1 nm	-	Circular area near locality *Cinque Terre* (Liguria). Presence on the seafloor of conventional weapons	MEDITERRANEAN PILOT BOOK VOL. 1C 1982

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Northern Tyrrhenian Sea	Coordinates: ϕ 44°00'.010N; λ 009°42'.130E Radius 0,8 nm	100	Circular area near locality *Cinque Terre* (Liguria). Presence on the seafloor of conventional weapons	MEDITERRANEAN PILOT BOOK VOL. 1C 1982
Central Tyrrhenian Sea	Coordinates of vertexes: A ϕ 38°53'.450N; λ 008°37'.340E B ϕ 38°51'.550N; λ 008°37'.340E C ϕ 38°51'.550N; λ 008°39'.980E D ϕ 38°53'.600N; λ 008°39'.980E	0-10	Teulada Cape (South Sardinia). Presence on the shoreline of unexploded ordnance	MEDITERRANEAN PILOT BOOK VOL. 1A 1982
Central Tyrrhenian Sea	Coordinates of vertexes: A ϕ 38°38'.220N; λ 008°34'.970E B ϕ 38°51'.310N; λ 008°34'.970E C ϕ 38°51'.310N; λ 008°43'.030E D ϕ 38°55'.380N; λ 008°43'.030E	0 - 50	Teulada Cape (South Sardinia). Presence on the seafloor of unexploded ordnance	MEDITERRANEAN PILOT BOOK VOL. 1A 1982
Central Tyrrhenian Sea	Coordinates: ϕ 39°47'.430N; λ 008°28'.620E Radius 1 nm	50	West Sardinia. Presence on the seafloor of unexploded ordnance	MEDITERRANEAN PILOT BOOK VOL. 1A 1982
Central Tyrrhenian Sea	Coordinates: ϕ 40°45'.280N; λ 014°05'.830E Radius 0,12 nm	50	Gulf of Naples, near Miseno Cape. Presence on the seafloor of unexploded ordnance. The area is probably the same as the one indicated by the US Army where a huge quantity of chemical weapons[56] were dumped after WWII. On the nautical charts, in the middle of the area a shipwreck is marked.	MEDITERRANEAN PILOT BOOK VOL. 1A 1982

[56] US Army Research, Development and Engineering Command, 2001. Off-shore disposal of chemical agents and weapons conducted by the United States. Historical database n° 26. 16 pp. http://www.dailypress.com/news/dp-02761sy0oct30,0,2199000.story

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Southern Adriatic Sea	Coordinates: A ϕ 41°39'.000N; λ 017°05'.000E B ϕ 41°34'.000N; λ 016°48'.000E C ϕ 41°59'.000N; λ 016°40'.000E D ϕ 42°04'.000N; λ 016°55'.000E	200 - 400	Area located about 35 nautical miles north of Molfetta where fishermen indicated they accidentally recovered chemical weapons loaded mainly with yperite	Interviews with fishermen
Southern Adriatic Sea	Coordinates: A ϕ 41°18'.800N; λ 017°14'.000E B ϕ 41°24'.000N; λ 017°14'.000E C ϕ 41°18'.800N; λ 017°04'.000E D ϕ 41°24'.000N; λ 017°04'.000E	300 - 500	Area located about 20 nautical miles from Bari, direction 45°, where fishermen indicated they accidentally recovered chemical weapons loaded mainly with yperite	Interviews with fishermen
Southern Adriatic Sea	Coordinates: ϕ 41°12'.740N; λ 016°57'.670E	110	Shipwreck probably loaded with chemical weapons	Interviews with fishermen
Southern Adriatic Sea	Coordinates: ϕ 41°13'.400N; λ 016°54'.480E	105	Shipwreck probably loaded with chemical weapons	Interviews with fishermen
Southern Adriatic Sea	Coordinates: ϕ 40°38'.020N; λ 018°10'.390E Radius 2 nm	100	Circular area 6 nautical miles eastward Brindisi where fishermen accidentally recovered conventional weapons	Interviews with fishermen
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 40°46'.250N; λ 018°10'.420E B ϕ 40°45'.540N; λ 018°11'.340E C ϕ 40°47'.670N; λ 018°14'.120E D ϕ 40°48'.380N; λ 018°13'.190E	150	Rectangular area 12 nautical miles from locality Torre Cavallo (Brindisi), direction northeast, where fishermen accidentally recovered conventional weapons	Interviews with fishermen
Southern Adriatic Sea	Coordinates: ϕ 40°59'.790N; λ 017°49'.410E Radius 1,5 - 2 nm	200 - 250	Circular area 15 nautical miles from the locality Torre Guaceto (Brindisi) where fishermen accidentally recovered conventional weapons	Interviews with fishermen

Location	Coordinates and dimensional data of area	Depth (meter)	Note	Source
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 41°35'.790N; λ 015°59'.040E B ϕ 41°35'.540N; λ 015°59'.270E C ϕ 41°35'.940N; λ 015°59'.850E D ϕ 41°35'.790N; λ 016°00'.030E	40	Rectangular area 3 nautical miles from Manfredonia, direction southeast, where fishermen accidentally recovered weapons loaded with white phosphorus	Interviews with fishermen
Southern Adriatic Sea	Coordinates of vertexes: A ϕ 40°57'.790N; λ 017°22'.090E B ϕ 40°56'.760N; λ 017°22'.090E C ϕ 40°57'.790N; λ 017°23'.410E D ϕ 40°56'.760N; λ 017°23'.410E	80	Rectangular area 3 - 4 nautical miles east of Brindisi where fishermen accidentally recovered conventional weapons	Interviews with fishermen
Southern Adriatic Sea	Coordinates: ϕ 40°09'.790N; λ 018°30'.800E Radius 0,5 nm	70	Circular area 1 – 1,5 nautical miles off Otranto harbour where fishermen accidentally recovered conventional weapons	Interviews with fishermen
Northern Adriatic Sea	Coordinates: ϕ 45°47'.263N; λ 013°32'.700E	0-10	Harbour of Monfalcone. In 2005 the Italian Navy and CETLI recovered and destroyed at sea about 150 artillery munitions of chemical weapons filled with mustard gas.	CETLI

5.5 The characteristics of chemical weapons

In general terms, the contents of chemical weapons vaporise at the time of the explosion because of their specific structure. Artillery and aerial bombs, the principal kinds of "chemical" armaments, are able to contaminate locations far away from the fire point. They are also able to transport a significant amount of CWA causing a large amount of damage to the enemy. Mines, grenades, drums and gas cylinders used to spray the CWAs on the battlefield and to load munitions, are also types of chemical weapons.

Artillery chemical bombs are generally made up by a coassial cylinder which contains the explosive charge, surrounded by a space loaded with the CWA. When the bomb is not armed a screw ring is situated at the nose of the bomb instead of the detonator.

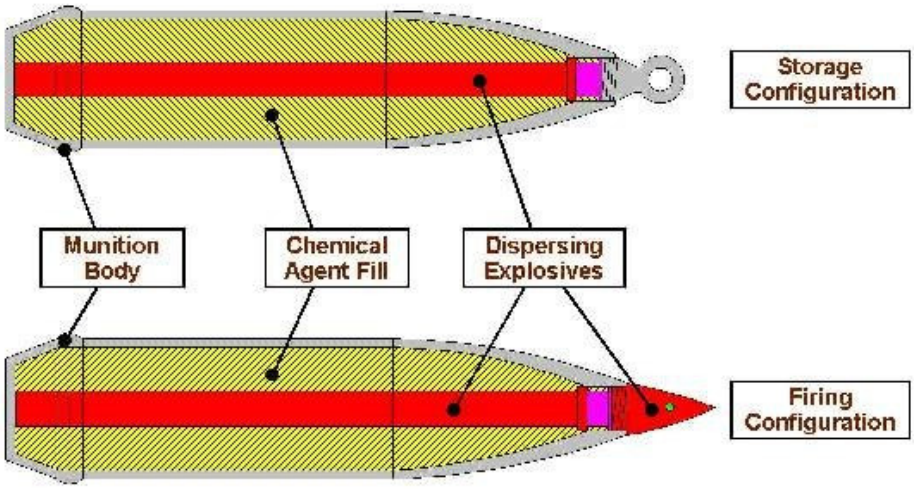


Fig. 5–32 Structure of a chemical artillery bomb[57]

Aerial bombs have the same coassial cylinder containing the explosive charge, surrounded by the CWA. The aerial bombs found on the Apulian sea bottom have often no vane, this is because it was taken off before loading them onto the dumping-ships or because they were stored with this configuration in the coastal depot.

[57] Mitretek Systems 2004b. *Chemical weapons and dissemination*. www.mitretek.org/home.nsf/EnvironmentEnergy/ChemBio#chem.

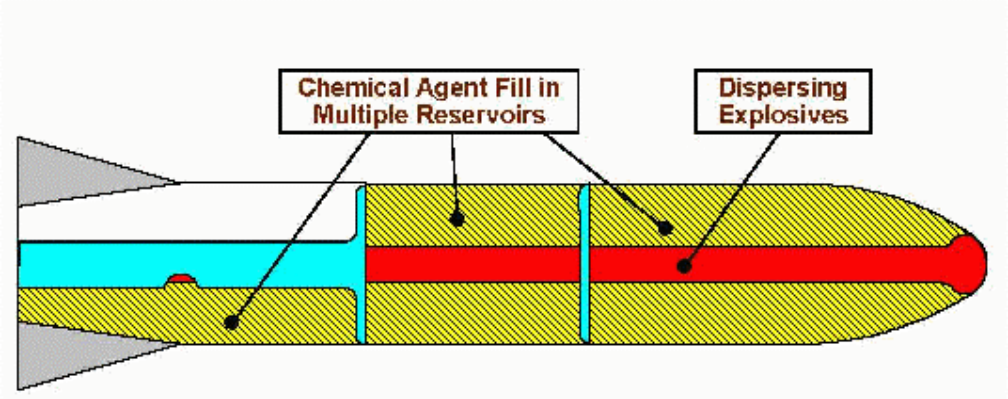


Fig. 5-33 Structure of a chemical aerial bomb [49]

In the Southern Adriatic Sea the dumping operations involved chemical munitions like hand grenades, aerial bombs, artillery bombs and mines. The CWA was also loaded in drums, used to fill the munitions before their employment. Most of these munitions have a thin casing (2 - 6 mm) in order to optimise the volume capacity available for the CWA. This special feature promotes the corrosive action by marine water and the CWA leakage.

Fig. 5–34 Rusted aerial bomb filled with mustard gas in S. Adriatic Sea, R.O.V. recorded during the A.C.A.B. project survey campaign (par. 4.4.1)

Fig. 5–35 Rusted aerial bomb M47 A1 recovered from the S. Adriatic Sea seafloor (source: State Archives in Bari)

Fig. 5–36 Rusted artillery bomb filled with mustard gas recovered by Italian Navy underwater operators in Molfalcone harbour (Northern Adriatic Sea) (source CETLI NBC)

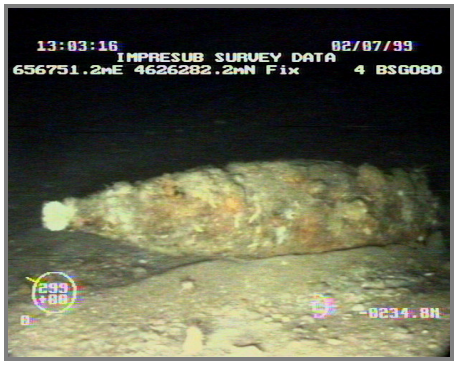


Fig. 5–37 Rusted artillery bomb filled with mustard gas in S. Adriatic Sea, R.O.V. recorded during the A.C.A.B. project survey campaign (par. 4.4.1)

The estimation of the corrosion rate takes into account a series of parameters not easy to measure. In fact besides the temperature, pH, salinity, hydrographic characteristics and O_2 measurement we should also consider the rusting characteristics of some materials such as mustard gas as well as the construction features of the bomb (drawings, kind and characteristics of metals used). The average rate of corrosion varies from 0,05 to 0,1 mm/year and it tends to decrease over time, probably because of the external oxidative layer which protects the shell. Then, when considering a chemical weapon with a thin casing of 3 mm the release of its contents could start after 30 years following its dumping at sea. The bomb will probably release all its contents when 50% of the corrosion process has occurred[58].

[58] NATO/CCMS/NACC -Pilot Study (1995). *Cross-Border environmental problems emanating from Defence-related installations and activities*. Summary Final Report - phase 1 1993 - 1995. Report n° 206

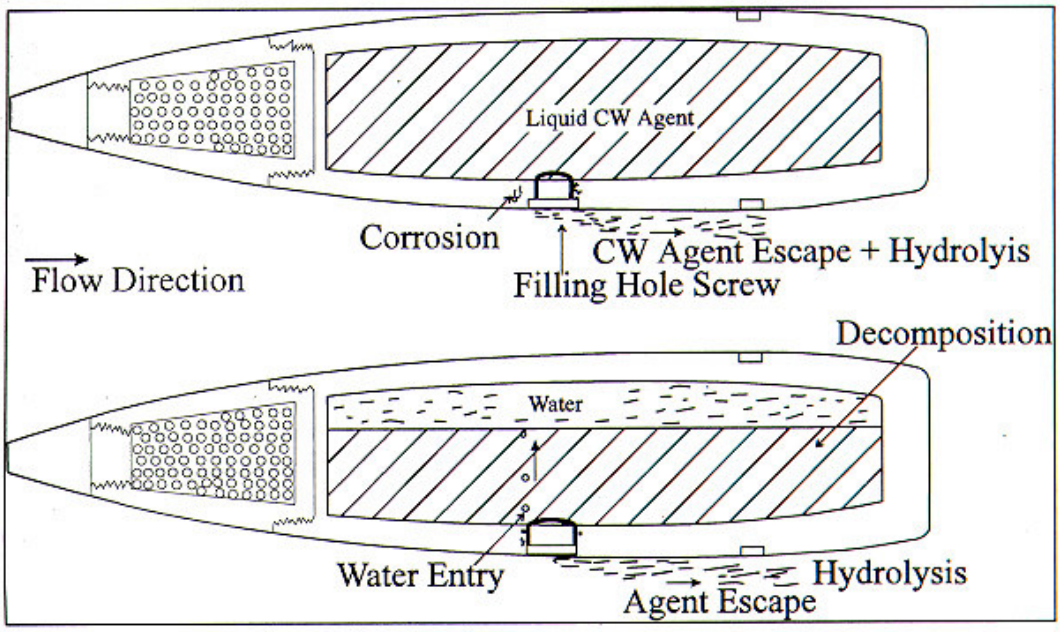


Fig. 5–38 Corrosion process of a bomb dumped at sea [50]

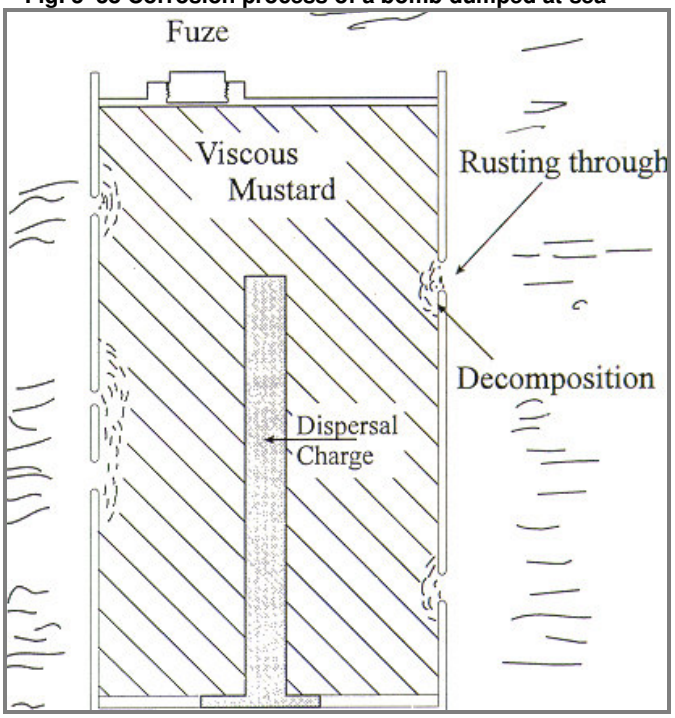


Fig. 5–39 Drum submitted to corrosion in seawater [85]

Bibliographical data concerning the main characteristics (dimension, shape, volume, weight and kind of CWA etc.) of munitions dumped in the Southern Adriatic Sea are summarised in Annex IV. The list is certainly incomplete due to

the difficulty in gaining information from the military personnel and archives consulted.

It is better to specify that the state of corrosion of the munitions observed in the Southern Adriatic Sea could compromise correct identification. In fact, corrosion has not only caused the alteration of some particular features (hooks, noses etc.) but it has also erased some of the colours and initials, which are often the only signs which distinguish a conventional weapon from a chemical weapon.

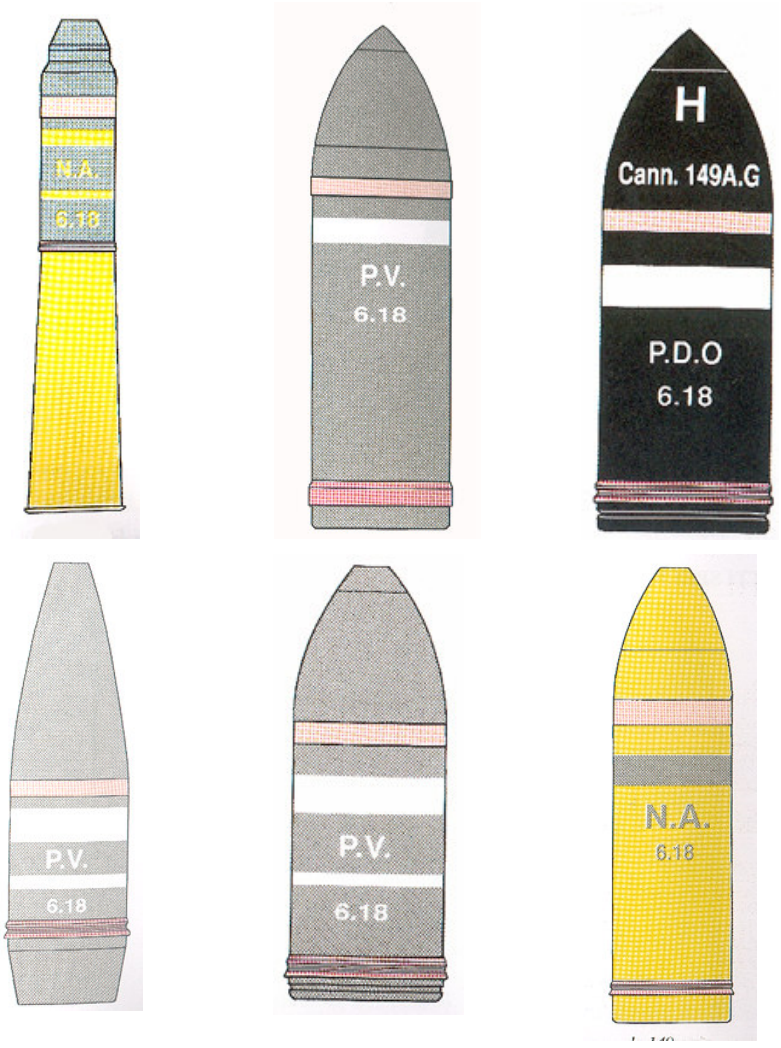


Fig.5–40 Some of the several types of chemical weapons produced during the First World War [59]

[59] Mantoan N., 2004. *La guerra dei gas (1914 – 1918).* Ed. Gaspari 120 pp.

Fig.5–41 A chemical weapon produced during WWI recovered by CETLI in a lake in the North-eastern Italian territory. A covering of anoxic sediment preserves the colours and initials, useful in identifying the contents of the material (source: CETLI)

5.6 Chemical Warfare pollutants

Chemical warfare agents are lethal compounds able to render temporarily uninhabitable large areas, which have been contaminated. From a strictly chemical point of view these compounds have a rather simple structure, they are mostly characterised by the presence of one or more halogen elements (e.g. chloride) as well as sulphur, arsenic, phosphorous, or cyanide.

These compounds are generally classified on the basis of the damage they may cause to the organism, they are as follows:

- Blistering agents: cause cell protoplasm destruction. Skin, eyes and mucous membranes of the respiratory apparatus are affected the most. Yperite and lewisite belong to this category;

- Suffocating agents: cause cell protoplasm destruction, inducing serious respiratory problems. Phosgene and diphosgene are typical suffocating agents[60].

- Irritant agents: cause severe irritation of mucous membranes. They are often divided in: vomitatory-sternutatory agents such as diphenyl arsine chloride (Clark I), diphenyl arsine cyanide (Clark II), adamsite (DN) and tear gas such as chloroacetophenone (CN) and chlorobenzalmalononitrile (CS)

[60] Kehoe R.A., 1943. *Pulmonary irritants.* Bull. N. Y. Acad Med. **19**: 340-355.

- Cellular Toxic agents: block endocellular oxidation processes. Carbon oxide and hydrogen cyanide belong to this group.
- Nerve agents: block nerve impulse transmission at the synaptic connection level. All nerve agents are known chemically as organo-phosphorus compounds. They are stable compounds, which can be easily dispersed and absorbed through the skin or by inhalation resulting in highly toxic, rapid effects. tabun (GA), sarin (GB), soman (GD) and "VX" belong to this group.

Another form of classification, adopted by the English army, separates the aggressive agents on the basis of their target organ. Furthermore a precise symbol is given to each category in order to facilitate their identification:


Affects the eyes (e.g. tear gas)


Affects the skin (e.g. yperite)


Affects the superior respiratory apparatus (e.g. Clark I)


Affects the lungs (e.g. phosgene)

In order to be utilized as war material, CWAs should be highly toxic and have the following properties:

- Physical properties which results in a high concentration of the substance in the place where it is being released;
- Easy to produce and at low costs;
- Long life stability;
- Not easily detectable in order not to facilitate enemy protection;
- Safe to use.

Through archive research, 23 different compounds used as CWAs in the ordnance dumped in the Southern Adriatic Sea have been identified; these include blistering agents, suffocating agents and blood toxic agents (annex V).

Through proper bibliographic researches, the identified compounds have been characterised from a chemical, physical and toxicological point of view (par. 5.6.1, 5.6.2, 5.6.3)[61] [62] [63] [64]. This information turned out to be very useful, in order to predict the behaviour of the compounds in the marine environment (Annex VI).

The behaviour of warfare agents in the marine environment is influenced by the physical properties of the agents. For instance, a warfare agent in viscous or highly viscous form or in lump form can be caught in nets. This cannot happen to substances in liquid or powder form. This is one reason why most accidents with warfare agents, so far, have involved viscous mustard gas[65].

5.6.1 Yperite

Yperite, also known as mustard gas or sulphur mustard (bis[2-chloroethyl]sulphide), is a blistering CWA. It is the main compound produced through the Levinstein process (particularly used in U.S.A. and U.K.) that produces several molecules with the common structure ClC_2H_4S-[66]. Impurity products derive from this chemical process. These constitute a polisulphur family, which shows the extremity of the molecule, the same structure ClC_2H_4S- observed in yperite. The most important is bis (2-chloroethyl)disulphide.

Mustard gas is an odourless and colourless liquid at room temperature, but the presence of degradation products (like solphoxides and solphones) gives rise to the blistering agent, a brownish colour and a characteristic garlic odour. In the table below the main physical - chemical characteristics of mustard gas are summarised.

[61] Franke C., Studinger G., Berger G., Böhling S., Bruckmann U., Cohors-Fresenborg D., Jöhncke U., 1994. *The assessment of bioaccumulation.* Chemosphere. **29**: 1501-1514.

[62] Lyman W.J., Reehl W.F., Rosenblatt D.H., 1990. *Handbook of chemical property estimation methods.* Washington, DC: Amer. Chem. Soc. p. 5-4.

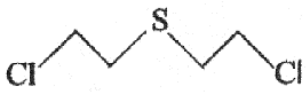
[63] Mabey W., Mill T., 1978. *Critical review of hydrolysis of organic compounds in water under environmental conditions.* Jour. Phys. Chem. Ref. Data **7**: 383-415.

[64] Romijn C.F.A., Luttik R., Meet D., Slooff W., Canton J.H., 1993. *Presentation of a general algorithm to include effect assessment on secondary poisoning in the derivation of environmental quality criteria. Part 1. Aquatic food chains.* Ecotoxicol. Environ. Saf., **26**: 61-85.

[65] Beddington J., Kinloch A. J., 2005. *Munitions Dumped at Sea: A Literature Review.*Imperial College London Consultants. 90 pp. www.imperial-consultants.co.uk

[66] Kinnear A.M., Harley-Mason J., 1948. *The composition of mustard gas made by the Levinstein process.* J. Soc. Chem. Ind., **67**: 107-110.

Table 5–2: main physical – chemical characteristics of yperite (mustard gas) [67]



C.A.S. number 505-60-2

C.A.S. name 1,1'-thiobis(2-chloroethane)

Odour	garlic	Solubility in water (20°C)	0.8-0.9 g/l
Colour	brownish (mustard)	Density	1.27 g/cm^3
Melting point (°C)	13-14 °C	Distribution coefficient Organ carbon/Water (LogK_{oc})	2.12
Boiling point (°C)	217°C	Distribution coefficient Octanol/Water (Log K_{ow})	1.37
Vapour pressure (20°C)	0.072 mmHg	Bioconcentration Factor $(BCF)_{fish}$	0.3

Mustard gas, when dispersed in the marine environment, is expected to sink (density 1.27 g/cm^3), to slowly melt in water (solubility 0.8-0.9 g/l) or to stick to sediment particles (Log K_{OC} value of 2.12 - distribution coefficient of a compound between organic carbon and water). On the seabed of the Southern Adriatic Sea, where the temperature is nearly 14 °C, yperite is present mainly as a liquid.

Although mustard gas has a low solubility in water, once it dissolves it quickly hydrolyses to primarily form thiodiglycol, together with other compounds including sulphonium and chloride salts (characterised by a polymeric structure) [68]. Fig 5–43. shows the main degradation pathway of mustard gas in saltwater, in Fig. 5–44 the main degradation pathway of bis (2-chloroethyl)disulphide (impurity product) is explained. Hydrolysis in seawater is two to three times slower than in fresh water, due to ions affecting the process. In addition, the rates of hydrolysis are slowed down at low temperatures. Depending on the

[67] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

Toxnet, 2001. U.S. National Library of Medicine, Bethseda, MD, USA, http://toxnet.nlm.nih.gov/.

Miretek Systems, 2004d. *Chemistry of Mustard Gas*. www.mitretek.org/home.nsf/homelandsecurity/Vesicants#mustard

U.S. Army Chemical Biological Defence Command. Edgewood Research Development and Engineering Center (ERDEC). *Material Safety data Sheet: Distilled Mustard (HD).* 28 February 1996. (www.cbdcom.apgea.army.mil).

U.S. Department of Health & Human Services, 2001. *Toxicological profile for "Mustard Gas".* Agency for Toxic Substances and Disease Registry (ATSDR).

Centro Tecnico Logistico Interforze NBC di Civitavecchia Centro Tecnico Logistico Interforze NBC, Civitavecchia - Italy (*p.c.*)

[68] Plunkett G., 2003. *Chemical Warfare Agent Sea Dumping off Australia.* Australian Government – Department of Defence. ISBN 0 642 29587 28 pp. www.hydro.gov.au/n2m/dumping/cwa/chemical.pdf

hydrodynamic conditions in the nearness of the seafloor, the production of sulphonium and chloride salts on the layer in contact with the seawater preserves the interior unaltered mustard gas for up to several years. In Figg. 5-21 and 5-34 this last situation is shown.

In conclusion, as the mustard gas leaks from the rusted bombshells, it should mix with the bottom boundary layer, should be diluted and hydrolysed near the dumping site and finally preserved by the external layer made of degradation products.

In rusty chemical weapons dumped at sea the observed mustard gas is often "viscous sulphur mustard". This is sulphur mustard to which a thickener has been added (polystyrene or wax). Its properties are therefore completely different from ordinary sulphur mustard and it behaves differently in the environment. Viscous sulphur mustard looks like wax; it is viscous and sticky. The thickener prevents dissolution and hydrolysis, allowing the formation of long-lasting plastic lumps on the seabed. This is the case in the Baltic Sea where fishermen with their fishing-nets have accidentally collected lumps of viscous sulphur mustard [69].

Fig. 5–42 Lumps of "viscous sulphur mustard" accidentally collected in the Baltic Sea[70])

[69] HELCOM CHEMU, 1994. *Report on Chemical Munitions Dumped in the Baltic Sea*, Report to the 16th Meeting of Helsinki Commission, 8 - 11 March 1994 from the Ad Hoc Working Group on Dumped Chemical Munition, Danish Environmental Protection Agency, http://www.helcom.fi/sea/Reportonchemicalmunitions.pdf

[70] Helsinki Commission (HELCOM), 2002. *HELCOM Manual on Co-operation in Response to Marine Pollution within the framework of the Convention on the Protection in the Marine Environment of the Baltic Sea Area (Helsinki Convention)*.**vol. 2.** www.helcom.fi

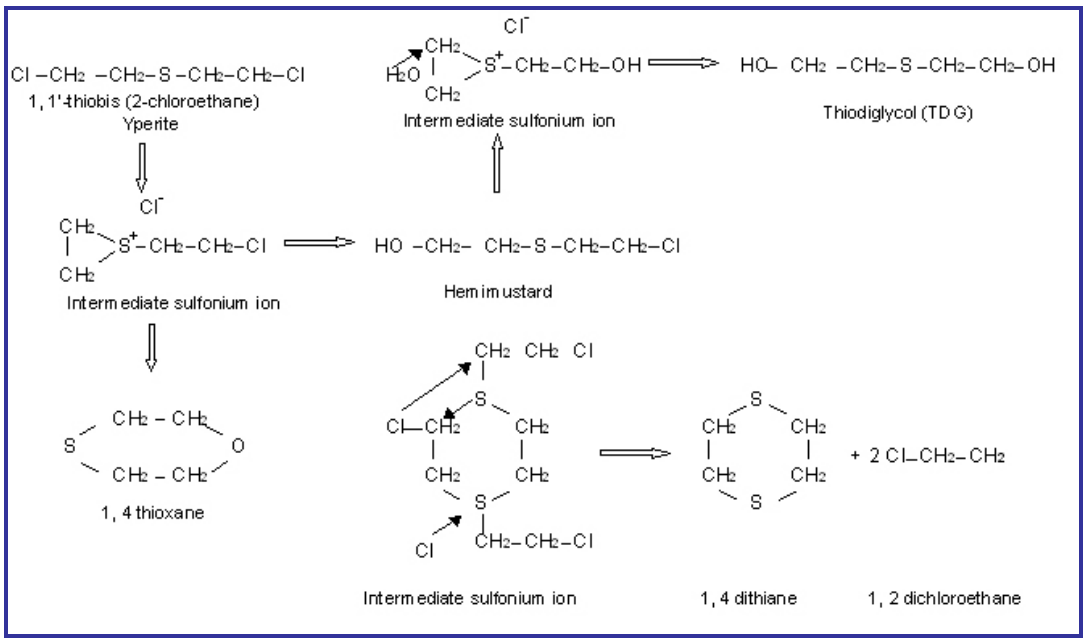


Fig. 5–43 Main degradation pathway of mustard gas in saltwater. Note that 1,4-thioxane and 1,4-dithiane are compounds revealed in samples of sediments collected in 1999 in the Southern Adriatic Sea CW dumping site during the A.C.A.B. project (also study area in the present project)

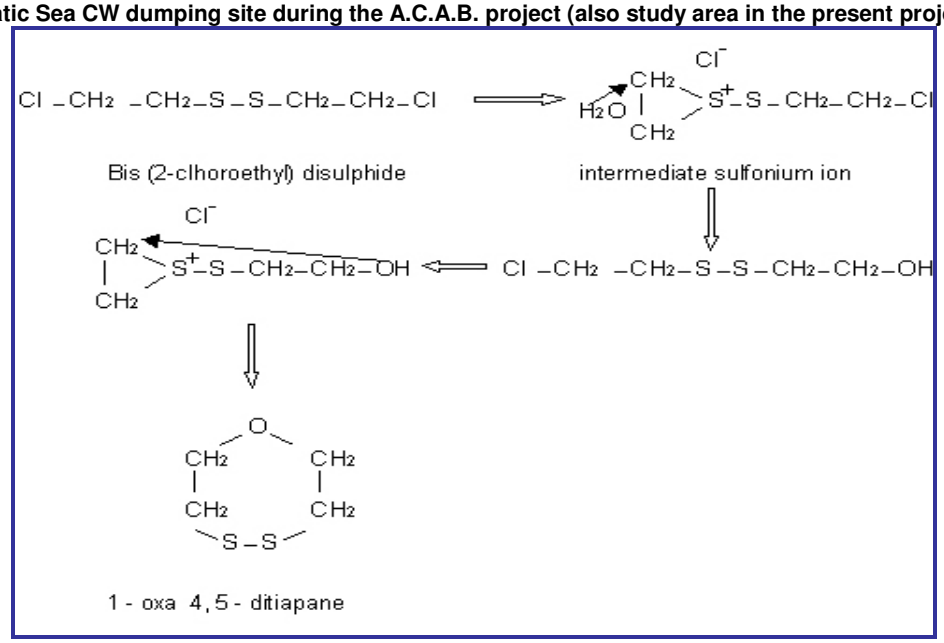


Fig. 5–44 Main degradation pathway of bis (2-chloroethyl)disulphide (impurity product) in saltwater. Note that 1-oxa-4,5-dithiapane and 1,2,5-trithiapane are compounds revealed in samples of sediments collected in 1999 in the Southern Adriatic Sea CW dumping site during the A.C.A.B. project (also study area in the present project)

Metabolism of yperite and its degradation products

The physiological effects and toxicities of yperite and its degradation products need to be more investigated, since the proposed mechanism of the cytotoxicity of sulphur mustard is based on the simplified S_N1 hydrolysis and is not fully understood.

Below the main reactions of yperite in the organism are reported. In addition to the potential contribution of sulphonium salts to the biologic activity of sulphur mustard, the oxidized forms of sulphur mustard (sulphoxide and sulphone) may also be of importance. The reactions of the sulphoxide are much slower than those of the sulphone leading to a detoxification mechanism (oxidation of sulphur mustard to its sulphoxide). The sulphone, on the other hand, is quite reactive via the elimination of HCl to form the divinylsulphone to which nucleophiles add (Fig. 5–45) [71].

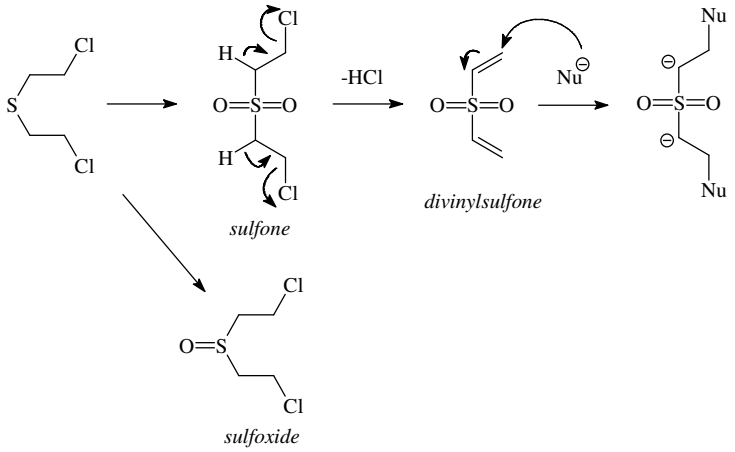


Fig.5–45 Production of divinylsulphone and sulphoxide from yperite

Sulphur mustard shows an high reactivity towards a wide range of nucleophilic species. Particularly, it reacts with sodium salts of alcohols (ethanol, methanol, etc.) to give ethers, but the yields are only fair; with the corresponding sulphur compounds, almost quantitative yields are obtained. With salts of organic acids, esters of thiodiglycol are produced (Fig. 5–46).

[71] Papirmeister B., Feister A.J., Robinson S.I., Ford R.D., 1991. Medical Defence Against Mustard Gas: Toxic Mechanism and Pharmacological Implications. Boca Raton, FL: CRC Press.

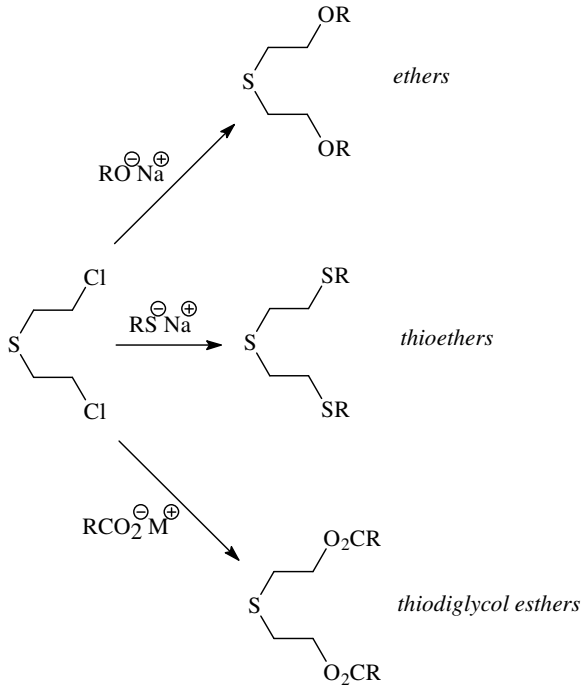


Fig. 5–46 Production of ethers and esthers from yperite

With ammonia and primary amines, a thiomorpholine derivative is formed. The reaction can occur readily also with secondary amines to give adducts of nucleophilic substitution of chloride, while quaternary ammonium salts are given from tertiary amines (Fig. 5–47).

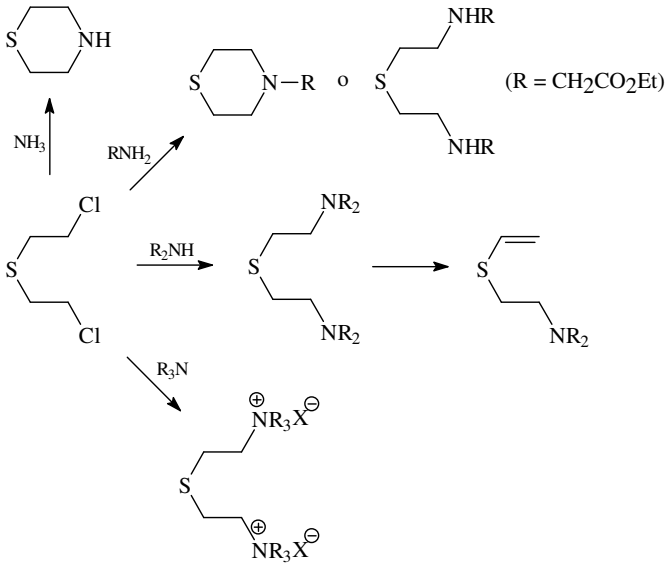


Fig. 5–47 Production of amines from yperite

Evidence that the cytotoxicity of sulphur mustard is due to the alkylation of DNA was obtained from studies with bacteria, DNA-containing bacterial viruses, and transforming DNA. The later discovery that the sensitivity of bacterial and mammalian cells is critically dependent on the cell's capacity for repairing sulfur mustard-induced DNA damage strongly supports the DNA target hypothesis. The relevance of DNA damage and repair to the vescicant action of sulfur mustard is supported by the observation that inhibitors of DNA repair significantly exacerbate skin injury [72] [73].

Sulphur mustard at neutral pH alkylates purines, pyrimidines, nucleosides and nucleotides, preferentially at N-7 of guanine (Fig. 5–48) and N-1 of adenine (Fig. 5–49). Reactions with O-6 and N-2 of guanine and N-6 of adenine have also been reported.

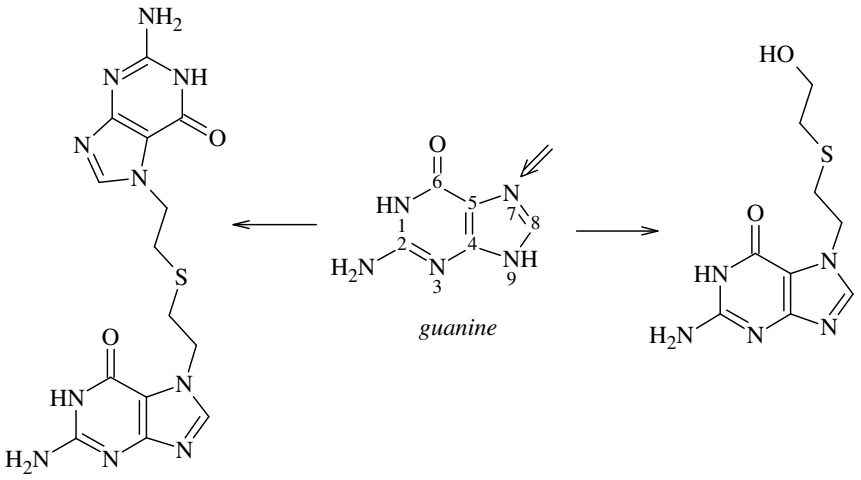


Fig. 5–48 Reaction of yperite with guanine (strand and interstrand link)

[72] Colvin M., Chabner B.A. 1990. *Alkylating Agents* in: Chabner B.A., Collins J.M., eds. Cancer Chemotherapy: Principles and practice. Philadelphia: J.B. Lippincott.

[73] Pechura C.M., Rall D.P. 1993. *Veterans at Risk. The Health Effects of Mustard Gas and Lewisite*. National Academy Press: Washington. 427 pp.

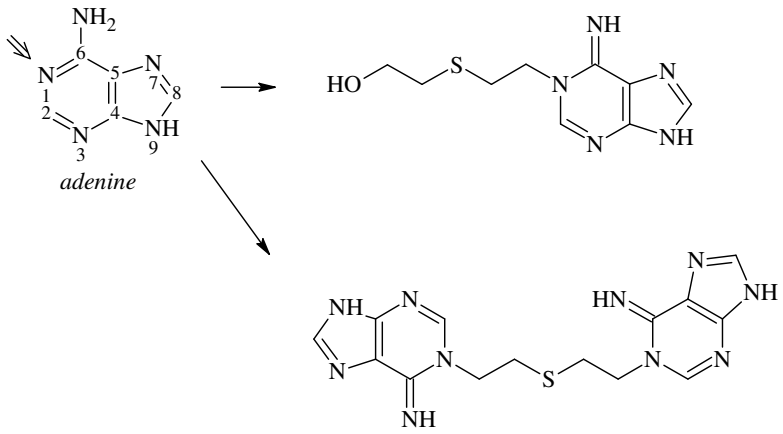


Fig. 5–49 Reaction of yperite with adenine (strand and interstrand link)

Sulphur mustard is more cytotoxic than its monofunctional analogue, because of its ability to form interstrand cross-links between guanines of the double helix, which prevents strand separation during replication. Furthermore, 7-alkylguanines and 3-alkyladenins of DNA are unstable and are released spontaneously from sulfur mustard-treated DNA at physiologic pH and temperature by cleavage of the N-9 glycosyl bond.

Although sulphur mustard also reacts with RNA, proteins, and phospholipids, the consensus of opinion has been for some time that it is the alkylation of DNA that is by the far the most important of its actions. The interstrand DNA cross-link produced by bifunctional mustard compounds is probably the lesion that produces lethality at the lowest frequency of occurrence and at the lowest concentration of the agent. However, cell death from this lesion is delayed for a number of hours, until the cell replicates its DNA or undergoes division.

At higher cellular exposures, mechanisms other than DNA cross-linking become important and produce more rapid cell death. The acute damage of the cornea, mucous membranes, and skin seen with sulphur mustard is probably generated by one or more of these other mechanisms.

One mechanism that may be involved in acute damage is nicotinamide adenine dinucleotide (NAD) depletion by the nuclear enzyme poly (adenosine diphosphoribose) polymerase, which is activated by DNA strand breaks produced by sulphur mustard. The enzyme cleaves NAD between nicotinamide and adenine diphosphoribose (ADP) and joins the ADP molecules into polymers of ADP-ribose, which are then linked to nuclear proteins and to the enzyme itself. The process depletes cellular pools of NAD, which is required for ATP

synthesis. The subsequent depletion of ATP rapidly produces loss of energy-dependent functions in the cell and results in cell death [74].

Other potential mechanism which can explain the acute effects of exposition to sulphur mustard is related to the rapid inactivation of sulfhydryl peptides, especially glutathione. These sulfhydryl compounds are critical to maintaining the appropriate oxidation-reduction state of cellular components. In particular, enzymes that maintain calcium homeostasis are sulfhydryl dependent, and sulfhydryl depletion may lead to elevated cellular calcium levels and cell death. Glutathione is also thought to be critical in reducing reactive oxygen species in the cell and preventing lipid peroxidation and loss of membrane integrity.

The alkylation of guanine at the O-6 position is likely primarily responsible for the mutagenic consequence of cellular exposure to sulphur mustard. Indeed, the DNA repair enzyme O-6-alkylguanine-DNA alkyltransferase does not act to repair these lesions [75]. Cytogenetic sensitivity of chromosomes to sulfur mustard, caused by alkylation reactions, has paralleled that of X-rays. Fishermen exposed to sulfur mustard through netting of leaky barrels of mustard agents dumped at sea after World War II have been found to have elevated sister chromatid exchange (SCE) frequencies in their peripheral blood lymphocytes [76]. These phenomena are well known and used to evaluate the body exposition to ionizing radiations. In short, sulfur mustard is genotoxic in a wide variety of cells, producing chromosome aberrations and gene mutations *in vitro* in a dose-related fashion, similar to the deterministic effects of ionizing radiations.

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[74] Berger N.A. 1985. *Poly(ADP)-ribose) in the cellular response to DNA damage*. Radiation Research **101**: 4-15.

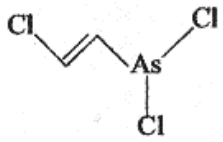
[75] Ludlum D.B., Kent S., Mehta J.R. 1986. *Formation of O^6-ethylthioethylguanine in DANN by reaction with the sulfur mustard, chloroethyl sulfide, and its apparent lack of repair by O^6-alkylguanine DNA alkyltransferase*. Carcinogenensis **7**:1203-1206.

[76] Wulf H.C., Aasted A., Darre E., Niebuhr E. 1985. *Sister chromatid exchanges in fishermen exposed to leaking mustard gas shells (letter)*. Lancet **1**: 690-691.

5.6.2 Lewisite

Lewisite (dichloro[2-chlorovinyl] arsine), like mustard gas, it is a blistering agent. Prof. W. Lee Lewis identified it for the first time in 1918 and coined the name of the substance. Pure lewisite is an odourless and colourless liquid at room temperature but the presence of degradation products gives rise to a substance with a yellow/brown colour and a characteristic geranium odour. The industrial process includes the production of the following mixture: cis and trans forms of lewisite, bis-(2-chlorovynil)chloroarsine, tris-(2-cholorovinyl)chloroarsine and arsenic trichloride [77].

Table 5–3: main physical – chemical characteristics of lewisite [78]



C.A.S. number 541-25-3

C.A.S. name (2-chloroethenyl)arsenousdichloride

Odour	geranium	Solubility in water (20°C)	0.5 mg/l
Colour	yellow/brown	Density	1.89 g/cm^3
Melting point (°C)	-18 °C	Distribution coefficient Organ carbon/Water (K_{oc})	n.d.
Boiling point (°C)	190°C	Distribution coefficient Octanol/Water (Log K_{ow})	n.d.
Vapour pressure (20°C)	0.394 mmHg	Bioconcentration Factor (BCF)	n.d.

The behaviour of lewisite in the marine environment is very similar to the behaviour shown by mustard gas. Lewisite is expected to sink (density 1.89 g/cm^3) and to melt slowly in water (solubility 0.8-0.9 g/l). Moreover, it is always in a liquid state in the marine environment. It hydrolyses quickly in contact with water producing oxides, hydroxides and their polymers that have the same toxicity level as lewisite. As the hydrolysis process continues, organic and inorganic arsenic

[77] Goldman M., Dacre J.C. 1989. *Lewisite: its chemistry, toxicology and biological effects.* Rev. Environ. Contam. Toxicol., **110**: 75-115.

[78] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

U.S. Army Chemical Biological Defence Command. Edgewood Research Development and Engineering Center (ERDEC). *Material Safety data Sheet: Lewisite.* 28 February 1996. (www.cbiac.apgea.army.mil).

Toxnet, 2001. U.S. National Library of Medicine, Bethseda, MD, USA, http://toxnet.nlm.nih.gov/.

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compounds are produced, like arsenious acid, obviously causing arsenic environmental pollution [79].

5.6.3 Other arsenic compounds

A family of arsenic compounds was produced for the first time at the end of WWI. The main ones are:

- adamsite (DM);
- diphenylchloroarsine (Clark I, DA);
- diphenylcyanoarsine (Clark II, DC)
- phenyldichloroarsine (PD).

These molecules are classified as irritants and vomitatory-sternutatory agents. They irritate eyes, nose and throat and usually cause headaches, nausea and vomiting. They can be lethal at high concentrations.

These compounds have often been mixed with other CWAs, as they are able to penetrate the activated carbon layer of gas masks. In this way both its tear and vomiting properties would force the victims to remove the mask, allowing the other noxious agents to act [80].

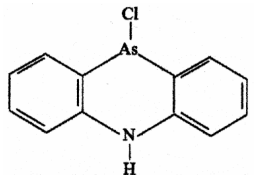
The German Army mixed the arsenic irritant agents with mustard gas, in particular, in order to produce the so-called "winter mustard" which maintains a liquid state even at low winter temperatures. The mixture is made of 49% mustard gas, 23% phenyldichloroarsine, 19% diphenylchloroarsine and 9% polystyrene used as thickening agents.

The physical – chemical properties of arsenic irritant agents are summarised in the table below.

[79] Bossle P.C., Ellzy M.W., Martin J.J., 1989. *Determination of Lewisite contamination in environmental waters by high-performance liquid chromatography.* Edgewood Arsenal, Aberdeen Proving Ground, MD. Report n. CRDEC-TR-042, January 1989, 13 pp.

[80] Miretek Systems, 2004e. *Chemistry of arsenical irritants.* www.mitretek.org/home.nsf/homelandsecurity/ChemistryofArsenicalIrritants

Table 5–4: main physical – chemical properties of Adamsite (DM) [81]



C.A.S. number 578-94-9

C.A.S. name 10-chloro-5-hydrophenarsazine

Odour	none	Solubility in water (20°C)	not soluble
Colour	yellow/green	Density	1.65 g/cm^3
Melting point (°C)	195	Distribution coefficient Organ carbon/Water (K_{oc})	5.75 x 10^3
Boiling point (°C)	410	Repartition coefficient Octanol/Water (Log K_{ow})	4.05
Vapour pressure (20°C)	negligible	Bioconcentration Factor (BCF)	262

An estimated K_{OC} value (distribution co-efficient between organic carbon and water) of 5.75 x10^3 indicates that adamsite will stick to sediments. Adamsite is practically insoluble in water. This agent hydrolyses very slowly, producing hydrochloric acid and bis(diphenylaminoarsine) oxide that are as toxic as adamsite. A measured log K_{OW} of 4.05 (distribution co-efficient between octanol and water) and an estimated BCF of 262 indicate some degree of bioaccumulation for adamsite. Even when adamsite is fully degraded, the product still contains an undegradable arsenic component that remains toxic. Since adamsite is not soluble in water, it has a higher density than water, and it sticks to sediments; it is expected to spread very slowly from the rusted bombshells lying on the seabed.

[81] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

Toxnet, 2001. U.S. National Library of Medicine, Bethseda, MD, USA, http://toxnet.nlm.nih.gov/.

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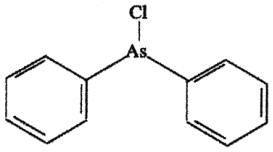
Table 5–5: main physical – chemical properties of Clark I (DA) [82]

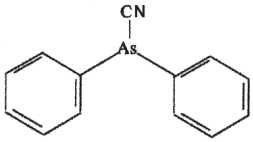


C.A.S. number 712-48-1

C.A.S. name Diphenyl arsine chloride

Odour	none	Solubility in water (20°C)	2 g/l
Colour	white/brown	Density	1.387 g/cm^3
Melting point (°C)	44	Distribution coefficient Organ carbon/Water carbon (K_{oc})	1.9x10^4
Boiling point (°C)	307	Repartition coefficient Octanol/Water (Log K_{ow})	4.52
Vapour pressure (20°C)	0.2016	Bioconcentration Factor $(BCF)_{extim.}$	505

Table 5–6: main physical – chemical properties of Clark II (DC)



C.A.S. number 712-48-1

C.A.S. name Diphenyl arsine cyanide

Odour	mix garlic/almond	Solubility in water (20°C)	2 g/l
Colour	white/pink	Density	1.33 g/cm^3
Melting point (°C)	30	Distribution coefficient Organ carbon/Water (K_{oc})	7.0x10^3
Boiling point (°C)	290	Repartition coefficient Octanol/Water (Log K_{ow})	3.29
Vapour pressure (20°C)	4.7x10^{-5}	Bioconcentration Factor $(BCF)_{extim.}$	68

On the basis of estimated K_{OC} values of 1.9x10^4 (Clark I) and 7.0x10^3 (Clark II), these compounds are expected to stick to sediments. Clark I and Clark II react very slowly with water and produce diphenylarsine and hydrochloric acid (Clark I), or cyanide (Clark II). Hydrochloric acid is neutralised by water, while cyanide is quickly broken down. Diphenylarsine is unstable in water and subsequently forms the highly stable tetraphenyl diarsine oxide. Information on tetraphenyl diarsine oxide is poor, although it is known to be a powerful warfare agent. Since Clark I

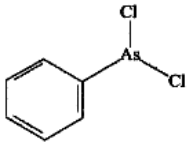
[82] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

Toxnet, 2001. U.S. National Library of Medicine, Bethseda, MD, USA, http://toxnet.nlm.nih.gov/.

Centro Tecnico Logistico Interforze NBC di Civitavecchia Centro Tecnico Logistico Interforze NBC, Civitavecchia - Italy (p.c.)

and Clark II are not soluble in water, they have a higher density than water and adsorb to the sediments, they are expected, like Adamsite, to spread very slowly from the rusted bombs lying on the seabed.

Table 5–7: main physical – chemical properties of PD



C.A.S. number 696-28-6

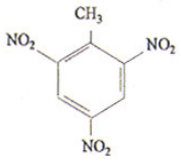
C.A.S. name Phenyl dichloro arsine

Odour	none	Solubility in water (20°C)	n.d.
Colour	none	Density	1.65 g/cm^3
Melting point (°C)	-16	Distribution coefficient Organ carbon/Water (K_{oc})	n.d.
Boiling point (°C)	252	Repartition coefficient Octanol/Water (Log K_{ow})	n.d.
Vapour pressure (20°C)	0.021	Bioconcentration Factor (BCF)	n.d.

5.6.4 trinitrotoluene (TNT)

2,4,6-trinitrotoluene (TNT) is the main component of explosives used in conventional ordnance. Other military explosives are RDX (cyclotrimethylenetrinitramine) and Tetryl (2, 4, 6 trinitrophenyl n-methylnitramin). TNT is a solid, yellow, odourless compound, produced by the combination of toluene, nitric and sulphuric acid mixture [83] .

Table 5–8: main physical – chemical characteristics of TNT [84]



C.A.S. number 118-96-7

C.A.S. name 2,4,6-trinitrotoluene

Odour	None	Solubility in water (20°C)	130 mg/l
Colour	Yellow	Density	1.65
Melting point (°C)	80.1	Distribution coefficient Organ carbon/Water (K_{oc})	300-1,100
Boiling point (°C)	240	Repartition coefficient Octanol/Water (Log K_{ow})	1.60
Vapour pressure (20°C)	negligible	Bioconcentration Factor (BCF)	209-453.

In the marine environment, TNT is a persistent and sinking polluting compound (low solubility and higher density than seawater). Its affinity with organic carbon (K_{oc} = 300-1,100) determines the natural trend of TNT to concentrate in sediments [85] [86].

Photolysis is the main and fastest degradation process of TNT in water [87] [88] [89] [90]. Various microorganisms have developed a slow degradation capacity in

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[84] Agency for Toxic Substances and Disease Registry (ATSDR). 1995. *Toxicological Profile for 2,4,6-trinitrotoluene (TNT)*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service

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[87] Howard PH, Boethling RS, Jarvis WF, Meylan, Michalenko, 1991. *Handbook of environmental degradation rates*. Chelsea, MI: Lewis Publishers: 454-455.

[88] Mabey WR, Tse D, Baraze A, Mill T, 1983. *Photolysis of nitroaromatics in aquatic systems: 1. 2,4,6-Trinitrotoluene*. Chemosphere, **12** (1): 3-16.

[89] Spanggord RJ, Mabey WR, Chou TW, Smith JH, 1985. *Environmental fate of selected nitroaromatic compounds in the aquatic environment*. In: Rickert DE (eds.). Chemical Industry Institute of Toxicology Series. Toxicity of nitroaromatic compounds. Washington D.C.: Hemisphere publishing corporation: 15-34

sediments, both in aerobic and anaerobic conditions[91] [92] [93]. Some plants are able to biodegrade the explosives as well; it appears that biodegradation is faster in anoxic and reducing conditions[94] [95] [96] [97].

The degradation pathway of TNT consists mainly in the reduction of nitro groups to aminic groups. The main compounds produced are: 2-amino-4,6-dinitrotoluene (2ADNT); 4-amino-2,6-dinitrotoluene (4ADNT) and 2,4- diamino-6-nitrotoluene (DAT). Another possible reaction is the loss of a nitro group from the TNT molecule and the subsequent production of 2,4 or 2,6-dinitrotoluene (DNT) (Fig. 5–50). In the nitro reduction process, the formation of 4ADNT is strongly favoured. The amines produced tend to bind to the clay fraction of sediments in an irreversible manner. These compounds, being rather reactive, may represent a greater risk to the environment than TNT itself [98] [99] [100].

In human beings TNT may be assumed with food, through the skin and through respiration[101]. As TNT enters the body it tends to concentrate in the liver, fat tissues and blood[102].

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[92] Hwang HM, Slaughter LF, Cook SM, Cui H, 2000. *Photochemical and microbial degradation of 2,4,6-trinitrotoluene (TNT) in a freshwater environment*. Bull Env Contam Tox, **65**: 228-235

[93] Oh BT, Sarath G, Shea PJ, Drijber RA, Comfort SD, 2000. *Rapid spectrometric determination of 2,4,6-trinitrotoluene in a Pseudomonas enzyme assay*. J Microbiol Met, **42**: 149-158

[94] Fernando T, Bumpus JA, Aust SD, 1990. *Biodegradation of TNT (2,4,6-trinitrotoluene) by Phanerochaete chrysosporium*. Appl Environ Microbial, **56** (6): 1666-1671

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[101] JOHNSON MS, VODELA JK, REDDY G, HOLLADAY SD, 2000. *Fate and the biochemical effects of 2,4,6-trinitrotoluene exposure to tiger salamanders (Ambistoma tigrinum)*. Ecotox Env Saf, **46**: 186-191.

[102] SABBIONI G, WEI J, LIU YY, 1996. *Determination of haemoglobin adducts in workers exposed to 2,4,6-trinitrotoluene*. J Chrom B, **682**: 243-248.

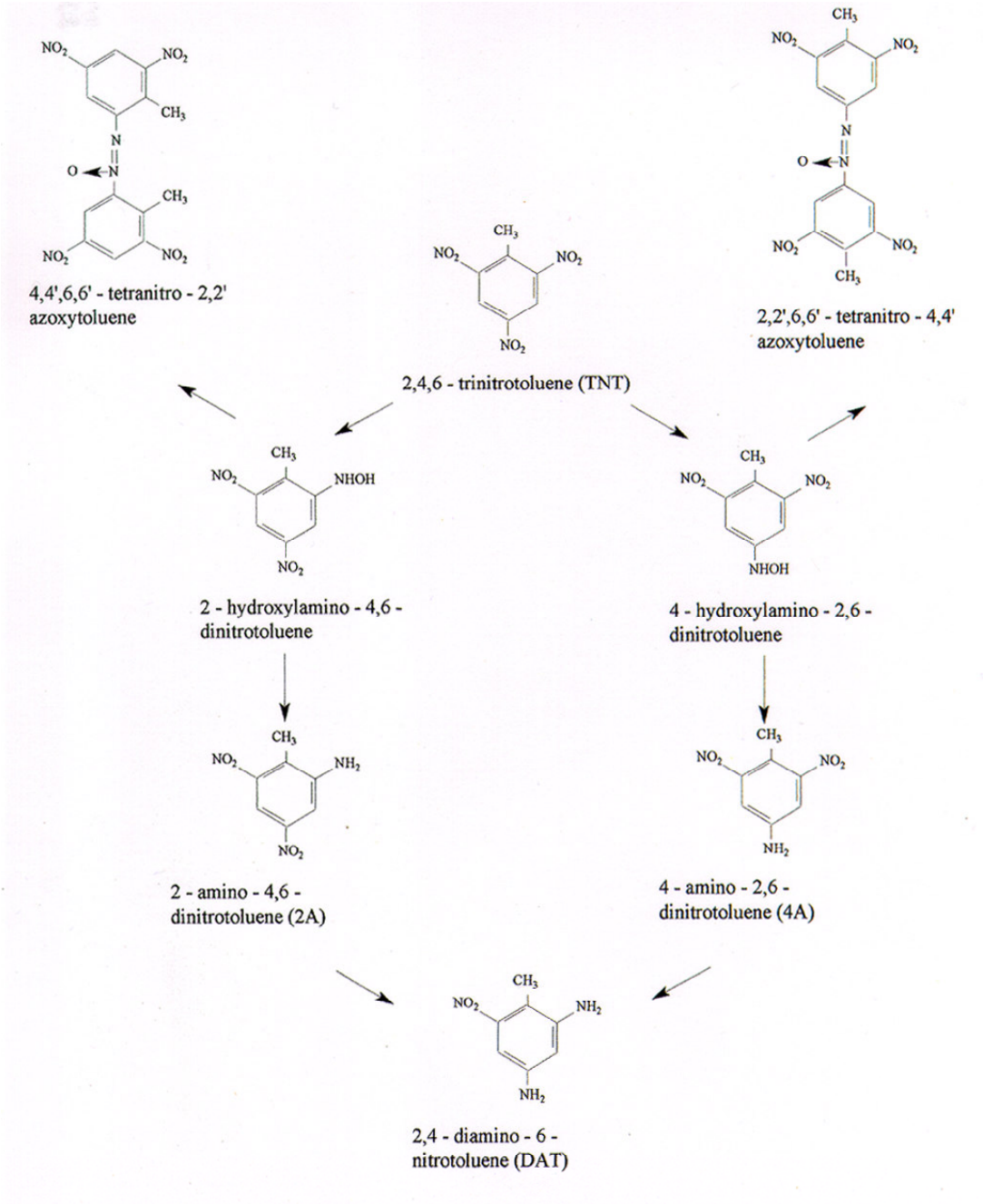

Fig. 5–50 Main degradation pathway of TNT

The main metabolic processes which determine the degradation of TNT take place within the liver, where the compound is rapidly reduced by means of enzymes (using the NADPH as an electronic donor) located on the microsomes. The process, which is catalysed by NADPH, cytochrome P450 redutase,

cytochrome P450 and phosphatidilcoline, leads to the production of 4ADNT and 2ADNT[103]. Most of the TNT and its degradation products are rapidly excreted[104]. These results are confirmed by Lotufo and Lydy[105] who observed a higher elimination rate than uptake rate of TNT and its degradation products in juvenile sheepshead minnows (*Cyprinodon variegates*) exposed in an aquarium to these compounds.

The mechanism through which TNT exerts its toxic effects is still rather unclear; nevertheless some hypotheses have been developed.

The amines deriving from the degradation pathway of TNT may link to the sulphydric groups of cellular proteins, altering their activity[97]. This mechanism would involve the liver's microsomial proteins NADPH-dependants and the oxyhemoglobin of the blood[106].

According to another theory, TNT and its degradation products would cause the creation of free radicals and consequently an oxidative stress to the organism[107] [108].

Several studies on workers in weapon production plants demonstrate that the toxic effects of TNT are exerted at different levels. In some cases anaemia and hepatitis are reported as the main consequences of TNT exposure[109], in others liver troubles with lethal effects are mentioned[110].

TNT has been classified as a potential carcinogenic agent for man as two researches on the chronic exposure in rats have shown a high number of carcinoma in urinary bladder as well as leukaemia and lymphoma in the spleen[111].

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[103] LEUNG KH, YAO M, STEARNS R, CHIU S-HL, 1995. *Mechanism of bioactivation and covalent binding of 2,4,6-trinitrotoluene*. Chem Biol Inter, **97**: 37-51.

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[111] ARMY, 1984a. *Determination of the chronic mammalian toxicological effects of TNT (twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the Fischer 344 rat). Final report: Phase III.*

Studies regarding the adverse effects of TNT on organisms have been mainly developed in the laboratory. Organisms living in salt, brackish or fresh water have been exposed to a known concentration of the contaminant for a certain length of time and the main results of some of these researches are summarised in the tables below (Tab. 5–9 and Tab.5–10).

Table 5–9: TNT and its degradation products, noxiousness in the aquatic environment.

Compounds	Test	Organisms	Effects observed[112]	Bibliography
TNT and 2A, 4A, 2,3 DAT	Development and survival of embryos	*Daphnia magna* (Crustacean) *Lytechinus variegatus* (Echinoderm)	1÷10 mg/l reduces the survival rate 1÷10 mg/l change the embryonic development	Davenport *et al.* 1994
TNT and TNTcc[113]	Biotoxicity test	*Pimephales promelas* (Crustacean)	EC_{50} TNT: 0,46 mg/l LC_{50} TNT: 2,58 mg/l EC_{50} TNTcc: 0,64 mg/l LC_{50} TNTcc: 1,6 mg/l	Smock *et al.* 1976
TNT	Biotoxicity test	*Lepomis macrochirus* (Osteyctes)	LC_{50}: 2,3-2,8 mg/l	Pederson 1970
TNT, 2A and 4A	Biotoxicity test with UV ray exposition	*Dugesia dorotocephala* (Planaria) *Daphnia magna* (Crustacean)	LC_{50} TNT: 1,56 mg/l LC_{50} 4A: 1,56 mg/l LC_{50} 2A: 2,57 mg/l LC_{50} TNT: 0,98 mg/l LC_{50} 4A: 1,31 mg/l LC_{50} 2A: 0,20 mg/l	Johnson *et al.* 1994
TNT	Growth rate alteration	*Selenastrum capricornutum* (Algae)	Growth rate alteration at conc. of 2,5 mg/l	Won *et al.* 1976
TNT	Growth and mortality rate alteration	*Neanthes arenaceodentata* (Polychetes)	$LOEC^2$: 0,12 mg/g LC_{50}:0,32 mg/g	Green *et al.* 1999
TNT	Growth and mortality rate alteration	*Leptocheirus plumulosus* (Anphipoda)	$LOEC^2$: 0,18mg/g LC_{50}:0,2 mg/g	Green *et al.* 1999
TNT	Acute effects on mortality rate	*Tigriopus californicus* (Copepoda) *Crassostrea gigas* (Bivalvia)	mortality 100% at conc. 10 mg/l mortality 100% at conc. 2,5 mg/l	Won *et al.* 1976

Contract no. DAMD-17-79-C-9120. Frederick, MD: U.S. Army Medical Research and Development Command, Fort Detrick. Document no. AD-A168 637.

ARMY 1984b. *Determination of the chronic mammalian toxicological effects of TNT (twenty-four month chronic toxicity/carcinogenicity study of trinitrotoluene (TNT) in the B6C3F1 hybrid mouse). Final report: Phase IV.* Contract no. DAMD-17-79-C-9120. Frederick, MD: U.S. Army Medical Research and Development Command, Fort Detrick. Document no. AD-A168 754.

[112] LC_{50} = conc. del composto sufficiente a provocare in 72 o 96 ore la morte del 50% degli organismi in esame calcolato in mg/l o in mg/g peso fresco

EC_{50} = conc. del composto sufficiente in 72 o 96 ore ad alterare il comportamento nel 50% degli organismi in esame

LOEC = minima concentrazione sufficiente ad alterare il comportamento degli organismi in esame

[113] TNTcc (*colored complex)* is a mix of secondary compounds of the TNT production process.

Table 5–9 (continue): TNT and its degradation products, noxiousness in the aquatic environment.

Compounds	Test	Organisms	Effects observed	Bibliography
TNT and its degradation products	Variation of biological diversity	Invertebrates community developed along a river down stream from a weapon production plant	Variation of biological diversity at conc. of TNT equal to 0,025 mg/l	Putnam *et al.* 1981
TNT	Survival and growth rate alteration	*Lemna perpusilla* (phanerogam)	Alteration at conc. of 1 mg/l	Palazzo and Leggett 1983
TNT	Survival rate alteration	*Anacystis aeruginosa* (Algae)	8 mg/l of TNT determines a 100% of mortality	Fitzgerald *et a*l. 1952
TNT	Biotoxicity test	*Artemia salina* (Crostacean)	LC_{50}: 29,1 mg/l	Toussaint *et al.* 1995
TNT	Biotoxicity test	*Lumbriculus variegatus* (Oligochetes)	LC_{50}: 4,9 mg/l	Bailey and Liu 1980

Table 5–10: TNT and its degradation products, noxiousness in the aquatic environment. Concentrations of TNT and its degradation products which result noxious or lethal for some aquatic species.

Organisms	Concentration	Bibliography
ALGAE		
Anacystis aeruginosa	EC_{50}: 4,1 mg/l LC_{50}: 8 mg/l	Fitzgerald *et al.* 1952
Selenastrum capricornutum	EC_{50}: 2,5 mg/l	Won *et al* 1976
PLANTS		
Lemma perpusilla	EC_{50}: 1 mg/l	Palazzo e Leggett, 1983
PLATELMINTHES		
Dughesia dorotocephfala	LC_{50}: 1,56÷2,57 mg/l	Johnson *et al.* 1994
OLIGOCHETES		
Lumbriculus variegatus	LC_{50}: 4,9-29mg/l	Bailey e Liu 1980; Liu *et al.* 1983
POLICHETES		
Neanthes arenaceodentata	LOEC: 0,12 mg/g LC_{50}: 0,32 mg/g	Green *et al.* 1999
CRUSTACEAN		
Artemia salina	29,1mg/l	Touissant *et al.* 1995
Daphnia magna	LC_{50}: 0,19÷11,7 mg/l	Liu *et al.* 1983
Leptocheirus plumulosus	LOEC: 0,18 mg/g LC_{50}: 0,2 mg/g	Green *et al.* 1999
ECHINODERMES		
Litechinus variegatus	1÷10 mg/l	Davenport *et al.* 1994
FISHES		
Pimephales promelas	LC_{50}: 1,2÷4,2 mg/l	Liu *et al.* 1983
Lepomis macrochirus	LC_{50}: 2,3÷3,4 mg/l	Pederson 1970; Liu *et al.* 1983
Salmo gairdneri	LC_{50}: 0,8÷2 mg/l	Liu *et al.* 1983
Ictalurus punctatus	LC_{50}: 1,6÷7,4 mg/l	Liu *et al.* 1983

These results confirm the potential risk to the biological communities due to the presence of explosive compounds within the marine environment and stress the need to study in depth the possible effects of conventional weapons lying on the seafloor of our seas.

5.7 Studies on environmental threats of CW dumped at sea

Besides the researches carried out by the partners in this project (see par. 5.7.1), only a few other studies have been performed regarding the environmental threats of chemical ordnance dumped at sea.

A research campaign supported by NATO in 1995, concerning the environmental damage deriving from military activities[114], took into consideration the effects of the dumped ordnance in the Baltic Sea. Even though it was not able to establish the state of conservation of the ordnance, the exact dumping sites, the behaviour of the chemical agents at sea nor their effects on the benthic ecosystems, the study states that, since the dumped ordnance does not seem to represent a serious hazard to the marine environment, any clean up operation would be superfluous .

This evaluation was made taking into account the hydrographic characteristics of the Baltic Sea, where the poor water exchange with the adjacent basins determines a clear water stratification which leads to anoxic conditions on the seafloor, preventing any form of benthic life.

The same conclusions are presented in a book which reviews the chemical weapons dumped at sea world-wide[115]. Taking into consideration the high costs of clean up operations as well as the high risk of contamination for the operators, it states that the best option is to leave the ordnance at sea. It has been estimated that for a hypothetical remediation activity in the Baltic Sea, where nearly 600,000 chemical weapons have been dumped, carried out using a properly equipped ship able to recover a maximum of 10 bombs per day, it would cost 100 billion dollars and would take 165 years of work. The study concludes that the main risk for both the marine environment and fishermen derives from the presence of yperite lumps on the seafloor.

During a Russian research program, which included 6 survey campaigns carried out between 1997 and 2001 in the North Sea and the Baltic Sea, two shipwrecks loaded with chemical weapons (Skagerrak strait in the North Sea and close to Bornholm island in the Baltic Sea, respectively) were investigated by a R.O.V.

[114] NATO/CCMS/NACC -Pilot Study (1995). Cross-Border environmental problems emanating from Defence-related installations and activities. Summary Final Report - phase 1 1993 - 1995. Report n° 206

[115] Surikov B.T., De vries I.J., Mikulin A.I., Kossy I.A., Holsboer J.H., Seward R.C., Duursma J.C., Heineken A.H., Duursma E.K., 1999. *Dumped chemical weapons in the sea: options.* Editor Prof. Dr. Egbert K. Duursma. 55 pp..

Another twelve were identified using a Side Scan Sonar coupled with a magnetometer[116].

The physical-chemical analysis performed by a CTD (Conductibility, Temperature and Depth), pH and O_2 meter, currentmeter, a sarin detector as well as the sediment samples collected, highlighted the following results:

- pH values of the waters surrounding the two investigated shipwrecks (1997 campaign) varied from 6.36 to 6.78, compared to natural values of 7.2-7.6;
- in the same waters phosphorous values reached 10g-at/l (2-5 times background values) due to the rather high number of phosphorous bombs contained in the wrecks;
- traces of sarin (nerve gas) were detected in a water sample collected in 2001 close to the wreck in the Skagerrak Strait;
- heterotrophic bacteria able to survive to yperite and its degradation products, isolated from the sediment samples collected near the Bornholm island and the Skagerrak Strait, were developed *in vitro.*

In 1998 the ship R/V "Professor Shtokman", utilised for the campaign, dredged a dark grey substance from the seafloor of the Baltic Sea, which was later identified as yperite[115].

A similar study was carried out in the waters NE of Ireland (Beaufort's Dyke explosives disposal site). A side scan sonar coupled with a magnetometer was utilised in order to detect the dumped targets. Sediment samples were collected with a Day Grab, whereas fish and molluscs were gathered using bottom fishing nets in order to detect traces of phosphorous, yperite, 2, 4, 6 trinitrotoluene (TNT), nitroglicerine, 1,3,5-trinitro-1,3,5-triazocicloesane (RDX) and methyl-2,4,6-trinitrophenylnitramine (tetryl)[117]. Although in this case laboratory analyses did not detect any trace of the target compounds, the authors highlighted the need to improve the analytical procedures and protocols.

[116] V. Paka and M. Spiridonov, 2002. An overview of the research of dumped chemical weapons made by the R/V "Professor Shtokman" in the Gotland, Bornholm & Skagerrak dump sites during 1997-2001. HELCOM MONAS 4/2002, Document 3/5/INF. http://www.helcom.fi/dps/docs.org

[117] Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD), 1996. *Surveysof the Beaufort's Dyke explosives disposal site, november 1995-july1996.* Fisheries Research Services Report No 15/96. 104pp..

In 2002 two research campaigns were carried out in the Skagerrak Strait in proximity to wrecks loaded with chemical weapons[118]. Four wrecks were investigated by a Remotely Operative Vehicle (ROV) and samples of sediment and water were collected using a multicorer and a Nansen water sampler respectively.

Laboratory analyses highlighted the presence of:

- yperite in one sediment sample (2.4 mg/kg d.w.);
- yperite' degradation products in sediment samples collected close to three wrecks. In particular 1,2,5-trithiapane; 1,4,5-oxadithiapane; 1,4,-dithiane; 1,4-thioxane were detected (the same compounds detected in the Southern Adriatic Sea (study area) during the A.C.A.B. survey campaign in 1999, par. 5.7.1);
- arsenic products such as Clark I, triphenylarsine and bis(diphenylarsine)oxide (degradation products of Clark I) in other sediment samples.

Arsenic products were detected the most, probably due to their higher stability. The mixture of arsenic products, known as "arsine oil", was usually added to yperite in order to develop a mixture ("*Winterlost*") which had the advantage of remaining liquid even at low temperatures.

In a study concerning the environmental aspects effected by chemical munitions dumped in the Baltic Sea, the potential threat of arsenic compounds to the benthic environment is calculated[119]. These compounds (Clark I and II and adamsite) hydrolyse to form molecules which contain arsenic and persist in seawater. Assuming that the Clark I and II and adamsite were dumped in the Baltic in the proportions that were manufactured in Germany (approx. 8% of the total) and the weight of the average amount of arsenic within them is approximately 27%, it can be calculated that the maximum amount of arsenic that may have been released into Baltic sea water from these weapons is approximately 280t. The natural concentration of arsenic in Baltic Sea water is approximately 1ppb. It can therefore be calculated that the maximum amount of arsenic that could be

[118] Tørnes J.A., Voie ø., Ljønes M., Opstad A.M., Bjerkeseth L.H., Hussain F., 2002. *Investigation and risk assessment of ships loaded with chemical ammunition scuttled in Skagerrak*. Project carried out by Forsvarets Forskningsinstitutt (FFI) on behalf of the Norwegian Pollution Control Authority (TA-1907/2002). 76 pp..

[119] Glasby, GP,, 1997. *Disposal of Chemical Weapons in the Baltic Sea*, In *The Science of the Total Environment*, **206**, pp 267-273.

released into Baltic Sea is just over 1% of the total amount of naturally occurring arsenic in the Baltic Sea water at any one time. Local enrichment of arsenic in the sediments would be possible. However, bioaccumulation of arsenic in marine organisms or enrichment in adjacent sediments above background level (100 ppm) has not been detected so far.

Finally, the ecological disaster off the Lenii Coast of the White Sea's Dvina Gulf is very well known. In May 1990, from 4 to 20 million specimens of the starfish *Asterias rubens* died[120] and even a young girl lost her life after having touched a beached starfish. The investigation subsequently carried out attempted to discover the most probable cause of such a disaster. Among the hypotheses was the presence of dumped chemical weapons loaded with yperite in the area: official data refers to 700 artillery bombs and to 5 tons of a mixture of yperite/lewisite contained in 31 iron cylinders. An official report of the Arkhangelsk Fishery Complex states that traces of yperite in samples of starfish, herring, mussels, seaweed, whitefish and flounder collected in the area in May - June 1990 were present [121].

5.7.1 Previous study in Southern Adriatic Sea

In this paragraph, all previous activities carried out by the participants to the RED COD project on the topic of environmental consequences of ordnance dumped in Southern Adriatic Sea are summarised.

The research started in 1997, when, after several parliamentary requests to the relevant Italian Ministries, the Ministry for the Environment requested that ICRAM carry out a pilot study in order to examine the situation regarding the dumping sites of chemical weapons in the Southern Adriatic Sea[122] [123]. To this end, ICRAM set up the A.C.A.B. (*Armi Chimiche Affondate e Benthos* = dumped chemical weapons and benthos) project. The priority tasks set were to identify the main CW dumping sites, to evaluate the state of the bomb shells and to assess the environmental risks related to the persistent CWAs.

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[120] Yufit S.S., Miskevich I.V., shtemberg O.N., 1996. *Chemical weapons dumping and the White Sea contamination*. Pp. 157-166. In: Kaffka A.V. (Ed), 1996. *Sea-dumped chemical weapons: aspects, problems and solutions*. Nato ASI series, 1. Kluwer Academic Publishers, Dordrecht, Boston, London, 170 pp.

[121] Alimov A. and Khebovich V., 1990. *What has happened in the White Sea*. PRAVDA, June 6 1990

[122] Amato E., Alcaro L., 1999. *A.C.A.B. Armi Chimiche Affondate e Benthos – Residuati bellici caricati con aggressivi chimici affondati in Basso Adriatico: distribuzione, stato di conservazione e conseguenze per gli ecosistemi marin*i. Final report, 2 volumes, 225 pages and 28 annexes. Italian Ministry of Environment.

[123] Amato E., Alcaro L., Corsi I., Della Torre C., Farchi C., Focardi S., Marino G., Tursi A., 2006. An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea). Marine Biology, **149** (1): 17-23.

In June 1999 a survey was carried out in one of the main dumping zones identified in the Adriatic Sea (see map of the Southern Adriatic Sea dumping areas, Annex II). A digital DATASONIC CHIRP SIS 1000 side scan sonar and sub bottom profiler coupled with a magnetometer (GEOMETRICS G880) were employed for the localisation of "targets" on the sea-bottom at depths in the range of 200 ÷ 300 m. The analysis of the recorded courses allowed the ranking of one hundred and two targets and the choice of the ones to consider as representative (Fig. 5–51).

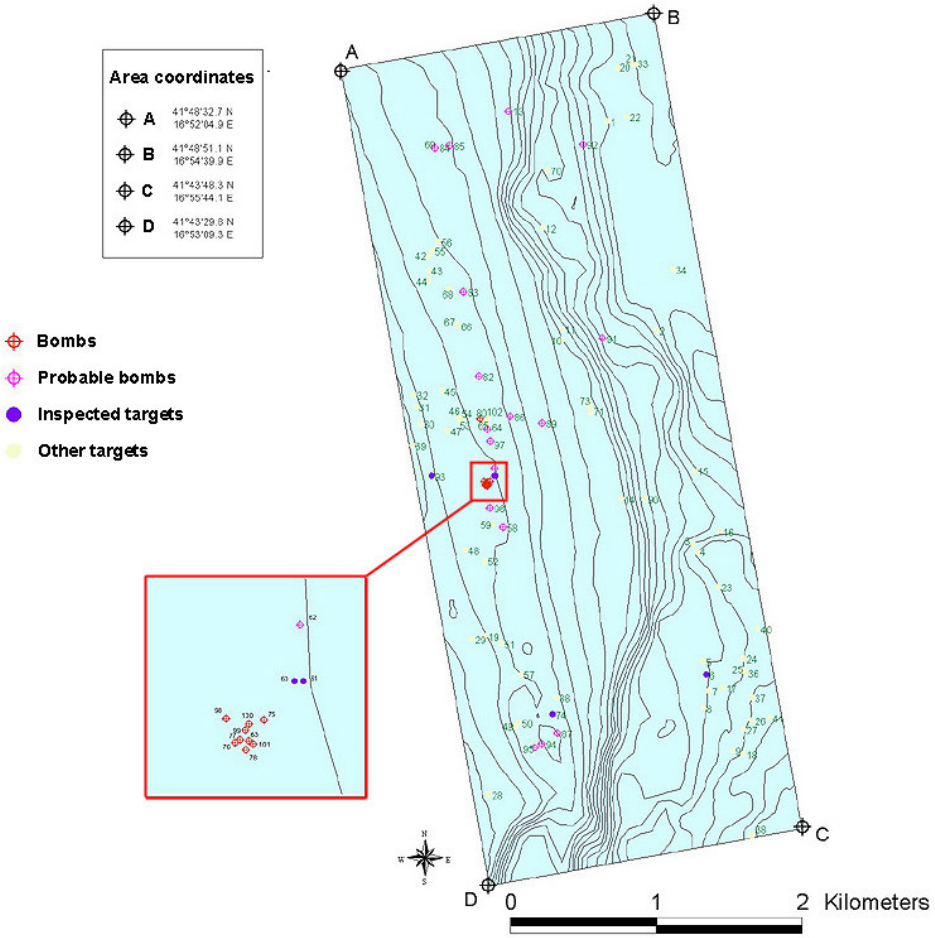


Fig. 5–51 Representation of one hundred and two targets observed during the A.C.A.B. survey campaign in 1999. The enlarged area, where several chemical artillery bombs were discovered, also represents the sampling campaign area of the present project where marine organisms have been collected (par. 5.2).

Fig 5–52 Artillery chemical bomb observed during A.C.A.B. survey campaign in the 1999

Fig 5–53 Water samples collected from ROV

Among the sixteen targets that were possible to observe by a remotely operated vehicle (SEA SURVEYOR SEA EYE 215), nine aerial and two artillery chemical bombs were identified (fig. 5–52). The R.O.V. was specially equipped to collect samples of sediment and water. In three cases, the CWAs contained in the observed rusted shells were clearly visible both from holes and fractures in the bomb's body, as well as on the surrounding seafloor (hard substrata made by coarse sediments enriched with fine particles).

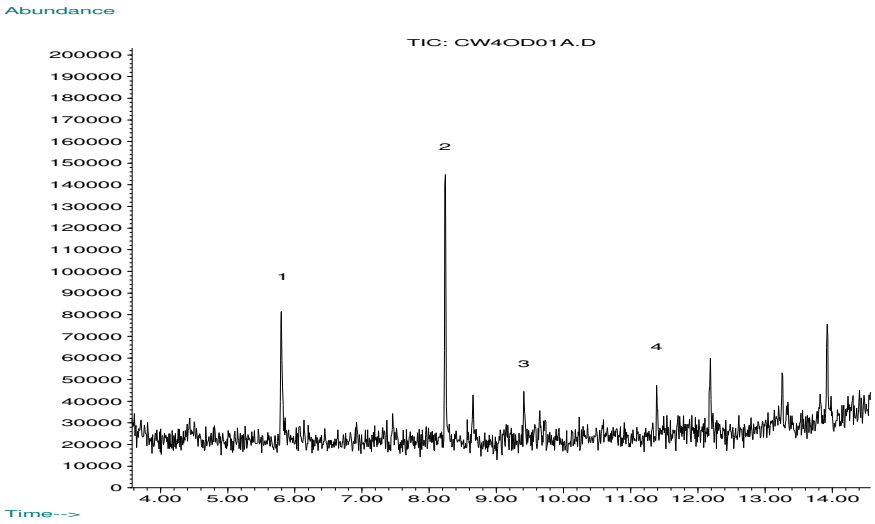
Sampling surveys were carried out to collect demersal fish both close to the observed bombs and in reference areas supposed to be unaffected by the dumping of war material. The laboratory analyses followed a similar

multidisciplinary approach applied to the present project. Water, sediments and fish tissues (muscle and liver) were exposed to four different analyses:

- arsenic, lewisite, yperite and their degradation product concentration;
- biotoxicity test;
- hystopatology;
- stress indexes.

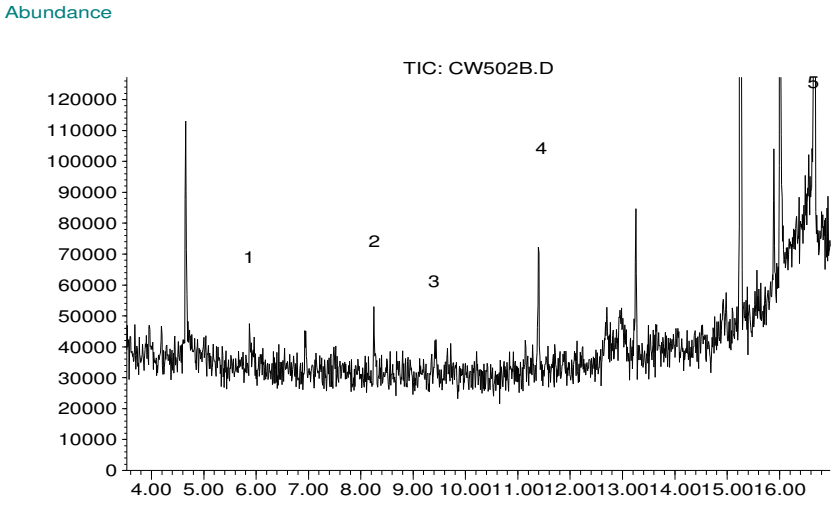
Sediments and fish tissues (muscle and liver) were analysed by GC-MS full scanning, in order to detect yperite and lewisite products traces.

The analyses carried out on the sediment samples collected near the ordnance showed the presence of several hydrolysis products of yperite and of bis-(2-chloroethyl)disulphide (impurity product of Levistein's process): 1,4-thioxane, 1,4-dithiane, 1-oxa-4,5-dithiapane and 1,2,5-trithiapane (Fig. 5–54 and Fig. 5–55).



1 1,4-thioxane
2 1,4-dithiane
3 1-oxa-4,5-dithiapane
4 1,2,5-trithiapane

Fig. 5–54: sediment sample (CW4) chromatogram



1 1,4-thioxane
2 1,4-dithiane
3 1-oxa-4,5-dithiapane
4 1,2,5-trithiapane
5 Sulphur (S_8)

Fig. 5–55: sediment sample (CW5) chromatogram

Tab. 5–11: 1,4-dithiane and 1,4-thioxane in two sediment samples

Sample	Compound	Concentration in the solution injected in the gas chromatograph (mg/l)	Ratio between volume of final solution and the dried weight of the sample (L/kg)	Concentration in the solid sample (ppm d. w.)
CW4	1,4-dithiane	**8.5**	**0.28**	**2.40**
CW5	1,4-dithiane	**4.7**	**0.28**	**1.30**
CW4	1,4-thioxane	**7.5**	**0.28**	**2.10**
CW5	1,4-thioxane	**2.9**	**0.28**	**0.81**

The arsenic concentration detected in four sediment samples collected near the ordnance is shown in the following table.

Tab. : As in sediment samples

Sample	As (ppm d. w.)
CW3	35.54
CW4	25.22
CW5	24.36
CW6	44.81

Yperite, lewisite and their oxidation and hydrolysis products were not detected in fish tissues. Samples of muscle and gills of *Conger conger* (Linnaeus, 1758), *Helicolenus d. dactylopterus* (Delaroche, 1809), *Raja asterias* Delaroche, 1809, *Raja clavata* Linnaeus, 1758, *Trigla lyra* Linnaeus, 1758 and *Trigla lucerna* Linnaeus, 1758 were analysed to detect traces of arsenic using AAS, showed, for the pilot area, values higher than the MPC (Maximum Permissible Concentration)

established in some countries (whose values vary from 0.1 to 6 ppm d.w.)[124] (Tab. 5–12, Tab. 5–13).

Tab. 5–12: concentrations of As in fish tissues collected in the pilot area (ppm d.w.)

Specimen	**Species**	**Muscle**	**Gills**
1/IP	Helicolenus dactylopterus	4.25	8.03
2/IP	*Helicolenus dactylopterus*	8.54	3.92
3/IP	*Helicolenus dactylopterus*	4.83	5.07
4/IP	*Helicolenus dactylopterus*	9.09	2.07
5/IP	*Trigla lira*	7.89	5.12
6/IP	*Helicolenus dactylopterus*	7.87	7.65
7/IP	*Helicolenus dactylopterus*	4.06	7.61
8/IP	*Helicolenus dactylopterus*	5.35	2.49
9/IP	*Helicolenus dactylopterus*	10.09	1.78
10/IP	*Conger conger*	15.9	4.49
11/IP	*Trigla lucerna*	3.91	3.19
12/IP	*Conger conger*	25.88	6.17
13/IP	*Raja asterias*	18.81	6.73
14/IP	*Trigla lucerna*	8.68	3.96
15/IP	*Trigla lucerna*	8.98	3.65
16/IP	*Raja clavata*	29.69	6.56
17/IP	*Raja asterias*	22.66	13.71
18/IP	*Trigla lucerna*	11.31	3.27

Tab. 5–13: concentrations of As in fish tissues collected in the Southern Tyrrenian Sea (Sicily) (ppm d.w.)

Specimen	Species	*Muscle*	*Gills*
1/C/IP	*Helicolenus dactylopterus*	1.64	2.06
3/C/IP	*Helicolenus dactylopterus*	1.04	4.01
6/C/IP	*Helicolenus dactylopterus*	1.57	2.87
8/C/IP	*Helicolenus dactylopterus*	1.21	/
10/C/IP	*Helicolenus dactylopterus*	1.67	/
12/C/IP	*Helicolenus dactylopterus*	1.81	2.36
13/C/IP	*Trigla lucerna*	2.61	3.23
14/C/IP	*Trigla lucerna*	1.90	/
15/C/IP	*Trigla lira*	2.29	/
16/C/IP	*Trigla lira*	2.67	/
17/C/IP	*Trigla lira*	2.66	/
18/C/IP	*Raja clavata*	3.89	/
20/C/IP	*Conger conger*	3.97	2.51

[124] Nauen C.E., 1983. Compilation of legal limits for hazardous substances in fish and fishery products. FAO Fisheries Circular 764. Food and Agriculture Organization of the United Nations. Roma

Particularly, the arsenic concentrations in muscle and gills of *Helicolenus d. dactylopterus* (Delaroche, 1809) showed to be significantly higher than the ones found in the same species collected at the control sites (Fig. 5–56).

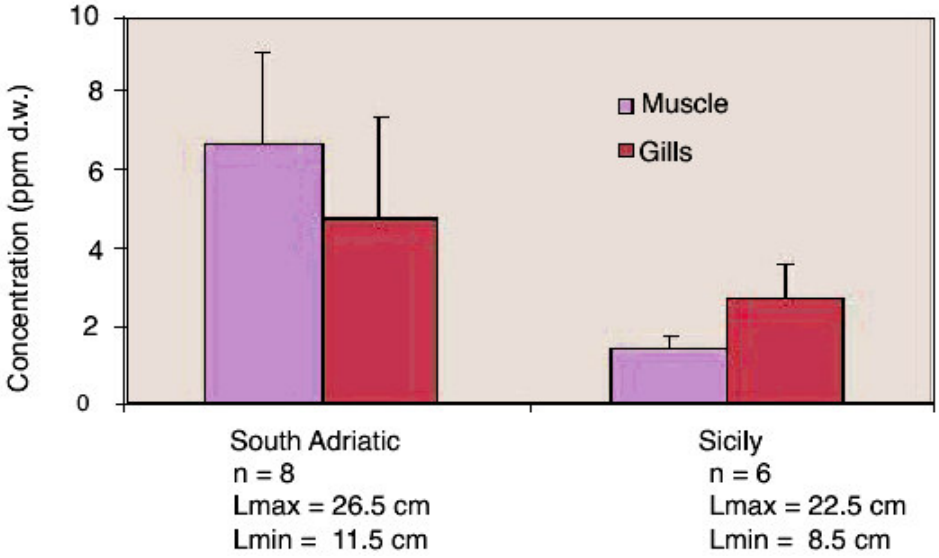


Fig. 5–56: concentration of As (ppm d.w.) in *Helicolenus d. dactylopterus* (Delaroche, 1809)

(Mann Withney *U* test, p<0.05)

Microtox® acute toxicity test was applied to sediment and water samples collected near the ordnance showing biotoxicity, both in the solid phase and in the interstitial water, in some of the samples (Tab. 5–14, Tab. 5–15, Tab. 5–16).

Tab. 5–14: Microtox® bioassay in water

Sample	Concentration (% v/v)	H% (light emission fall)	Result
CW1	90	<0	No acute toxicity
CW2	90	<0	No acute toxicity
CW4	90	<0	No acute toxicity
CW5	90	<0	No acute toxicity
CW6	9	46	Low acute toxicity

Tab. 5–15: Microtox® acute bio toxicity test in interstitial water

Sample	EC50 30' (% v/v)	Toxic Units (TU)
CW3	87.04	1.15
CW4	28.95	3.45
CW5	43.09	2.32
CW6	No acute toxicity	

Tab. 5–16: Microtox® acute bio toxicity test in sediments (Microtox® Solid Phase)

Sample	EC50 30' (% v/v)	Toxic Units (TU)
CW3	9.660	2.04
CW4	4.415	4.47
CW5	0.017	1165.80
CW6	6.223	3.17

Liver samples of *Conger conger, Helicolenus d. dactylopterus, Raja asterias, Raja clavata, Trigla lyra* and *Trigla lucerna* were analysed to measure the induction of CYP-450 1A (EROD, 7-ethoxyresorufin-O-deethylase) whereas the inhibition of Acetyl cholinesterase activity was measured in samples of brain (AchE b) and muscle (AchE m) (Tab. 5–17).

Tab. 5–17: biomarkers measured in fish collected both in the study and the control area

	EROD **(pmol/min/mg prot)**	**AChE m (pmol/min/g muscle)**	**AChE b (pmol/min/g brain)**
Pilot area			
Helicolenus dactylopterus	16.454 ± 8.085	4.482 ± 0.34	37.628 ±22.8
Trigla lira (n=1)	26.944	n. d.	n. d.
Conger conger	269.875 ± 24.96	12.137	14.226 ± 10.05
Trigla lucerna	95.421 ± 7.48	2.756 ± 0.18	3.431 ± 2.07
Raja asterias (n=1)	0.965	1.415	18.201
Raja clavata	0.570 ± 0.4	4.019 ± 3.85	18.120 ± 6.24
Control area			
Helicolenus dactylopterus	8.054 ± 5.87	6.790 ± 2.82	64.610 ± 33.23
Conger conger (n=1)	78.719	13.200	72.870

AChE cerebral activity in South Adriatic fishes shows significant interspecific differences: *Helicolenus d. dactylopterus* (Delaroche, 1809) specimens are the most sensitive bioindicators (37.628 nmols/min/g brain). In this species EROD values obtained from pilot individuals show a 50% increase of activity compared to the controls. Both the enzyme activity involved in detoxifying processes in liver tissues (EROD) and the physiological activity of enzymes in brain and muscle tissues (AChE) of individuals collected in the pilot area showed significant differences ($p<0.05$) compared to the controls values (Fig. 5–57, Fig. 5–58 and Fig. 5–59).

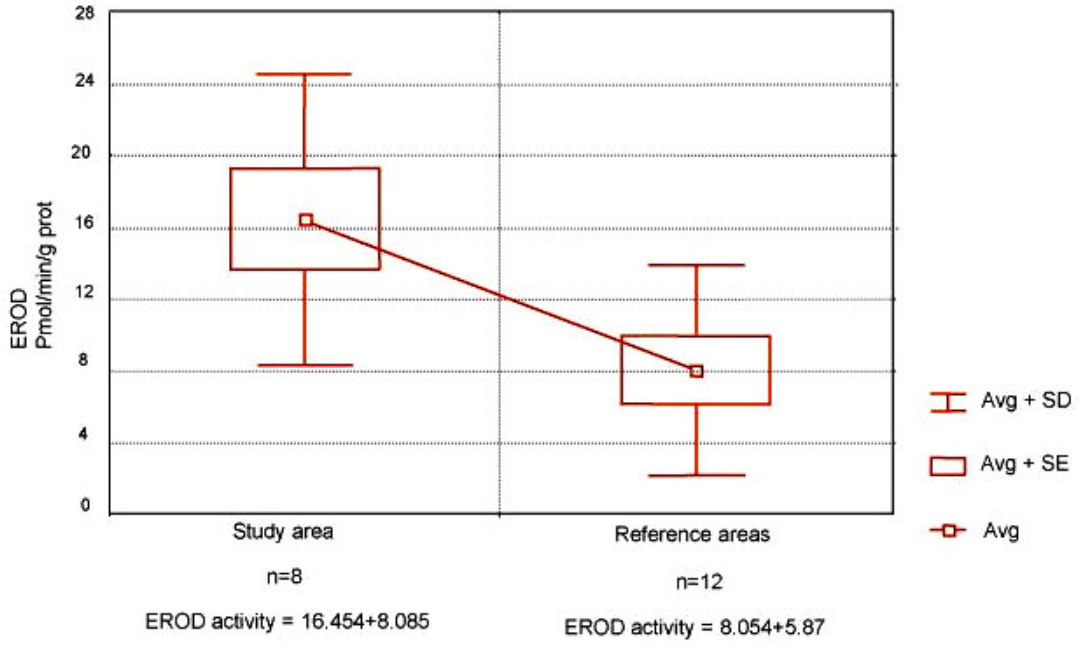


Fig. 5–57: EROD activity in specimens of *Helicolenus d. dactylopterus* (Delaroche, 1809) collected in the two areas

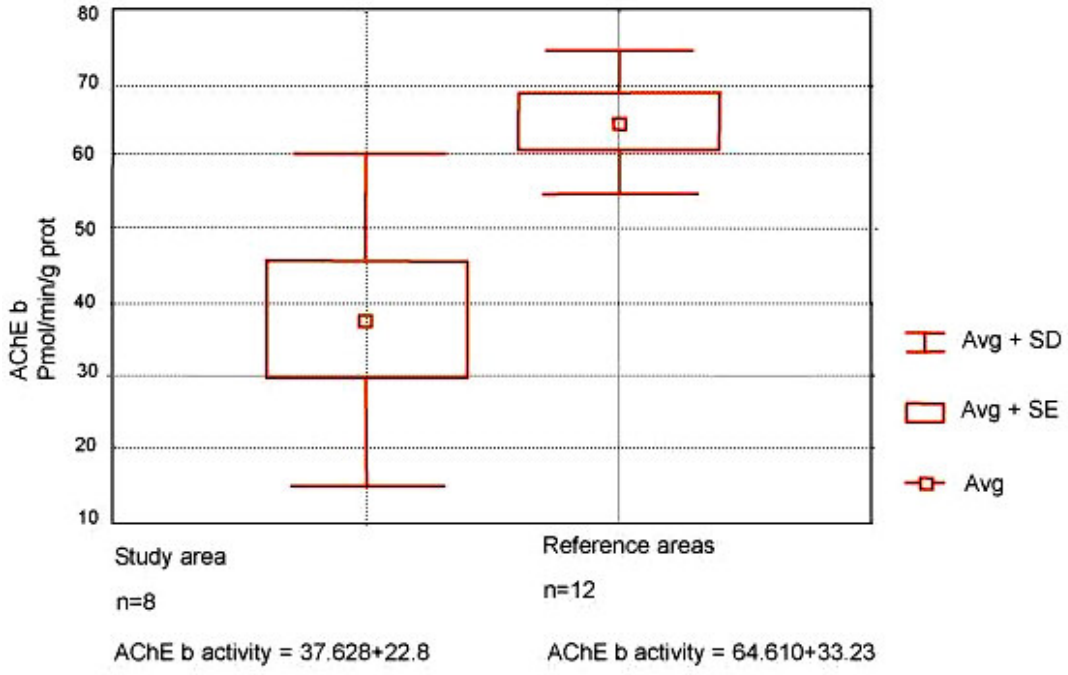


Fig. 5–58: cerebral AChE activity (pmol/min/g tissue) in specimens of Helicolenus d. dactylopterus (Delaroche, 1809) collected in the two areas

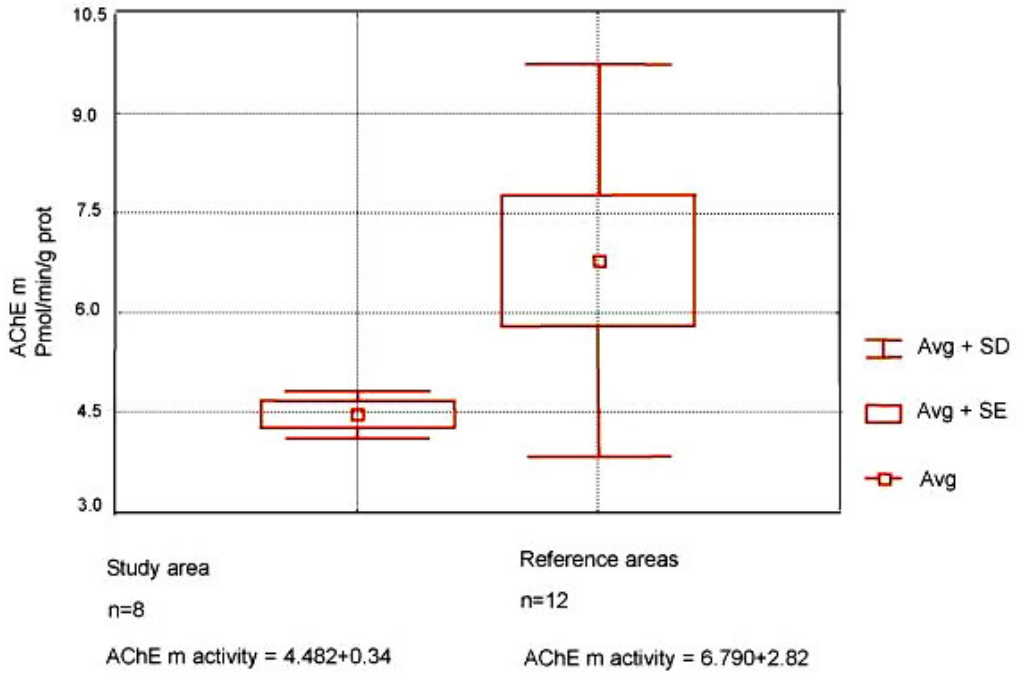


Fig. 5–59: muscular AChE activity (pmols/min/g tissue) in *Helicolenus d. dactylopterus* (Delaroche,1809) specimens collected in the two areas

Furthermore, a Health Assessment Index (HAI)[125] was assigned to each individual, according to the number of macroscopic alterations observed (Tab. 5–18, Tab. 5–19, Fig. 5–60, Fig. 5–61 and Fig. 5–62). Hystopatological analyses were performed on livers and spleens of the same fish. The HAI counted fifteen out of sixteen individuals of *Helicolenus d. dactylopterus* (Delaroche, 1809) as damaged whilst in the control site, only eleven out of twenty-one specimens of *H. dactylopterus* (Delaroche, 1809) showed significant macroscopic alterations. Histological analysis revealed evident damage (steatosis, fibrosis, granuloma, and atrophy of lymphatic centres) to liver and spleen tissues of sixteen out of eighteen *H. dactylopterus* (Delaroche, 1809) individuals analysed (Fig. 5–60). Table 5–18 shows the values of the Health Assessment Index.

[125] Adams S.M., Brown A.M., Goede R.W., 1993. A quantitative health assessment index for rapid evaluation of fish condition in the field. Transaction of the American Fish. Soc. **122**: 63-73.

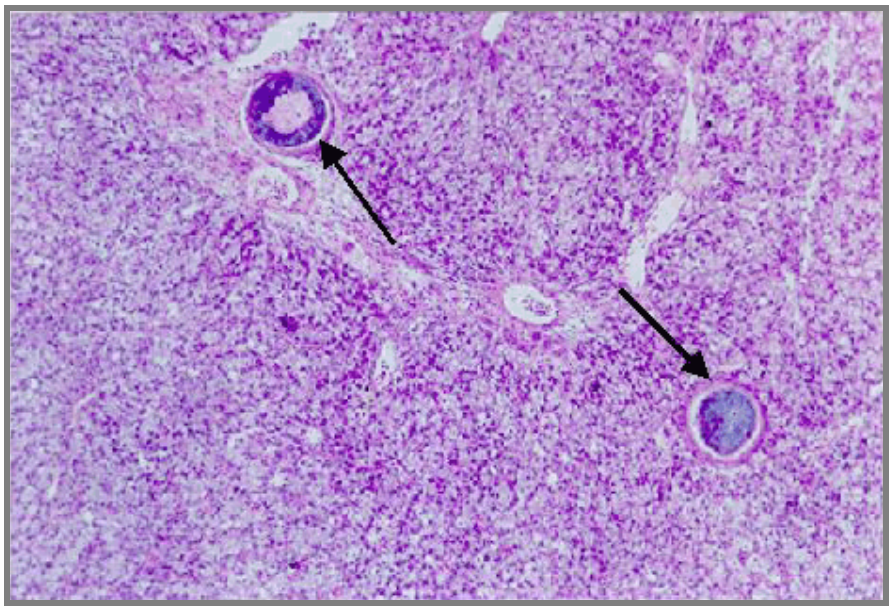


Fig. 5–60 *H. dactylopterus* (Delaroche, 1809) (sample 12/IP): hepatic granulomas (EE 10x)

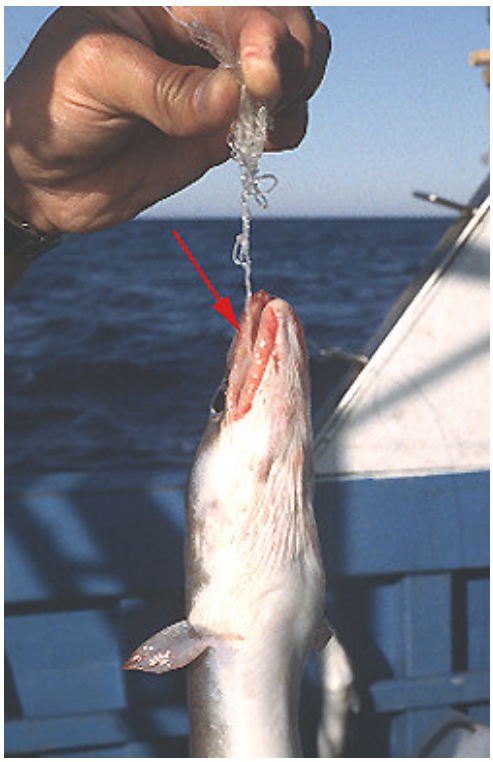


Fig. 5–61 *Conger conger* (Linnaeus, 1758). Coetaneous lesions on both sides of the mouth

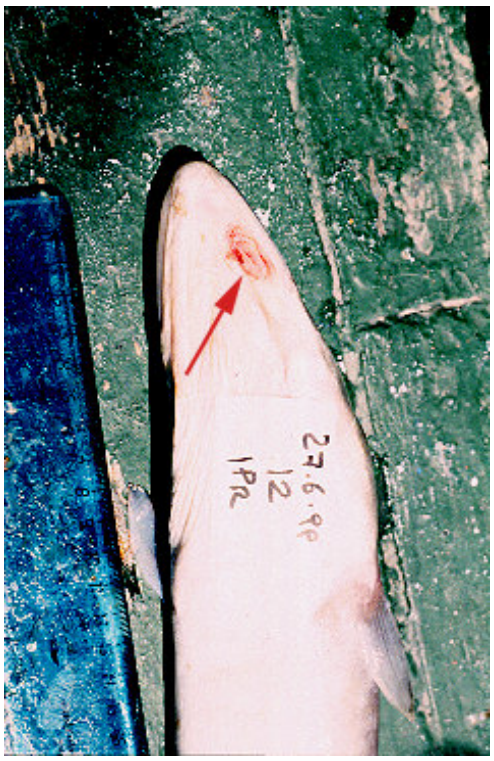


Fig. 5–62 *Conger conger* (Linnaeus, 1758). Submandibolary ulcer

Tab. 5–18: Health Assessment Index (HAI)

South Adriatic			South Tyrrenian		
Specimen	Species	HAI	Specimen	Species	HAI
1/IP	Helicolenus dactylopterus	120	1/C/IP	Helicolenus dactylopterus	30
2/IP	*Helicolenus dactylopterus*	10	2/C/IP	*Helicolenus dactylopterus*	0
3/IP	*Helicolenus dactylopterus*	30	3/C/IP	*Helicolenus dactylopterus*	0
4/IP	*Helicolenus dactylopterus*	90	4/C/IP	*Helicolenus dactylopterus*	0
5/IP	*Trigla lira*	70	5/C/IP	*Helicolenus dactylopterus*	0
6/IP	*Helicolenus dactylopterus*	60	6/C/IP	*Helicolenus dactylopterus*	60
7/IP	*Helicolenus dactylopterus*	0	7/C/IP	*Helicolenus dactylopterus*	30
8/IP	*Helicolenus dactylopterus*	20	8/C/IP	*Helicolenus dactylopterus*	0
9/IP	*Helicolenus dactylopterus*	60	9/C/IP	*Helicolenus dactylopterus*	30
10/IP	*Conger conger*	50	10/C/IP	*Helicolenus dactylopterus*	30
11/IP	*Trigla lucerna*	30	11/C/IP	*Helicolenus dactylopterus*	90
12/IP	*Conger conger*	50	12/C/IP	*Helicolenus dactylopterus*	30
13/IP	*Raja asterias*	60	13/C/IP	*Trigla lucerna*	0
14/IP	*Trigla lucerna*	90	14/C/IP	*Trigla lucerna*	0
15/IP	*Trigla lucerna*	60	15/C/IP	*Trigla lira*	0
16/IP	*Raja clavata*	0	16/C/IP	*Trigla lira*	0
17/IP	*Raja asterias*	0	17/C/IP	*Trigla lira*	0
18/IP	*Trigla lucerna*	30	18/C/IP	*Raja clavata*	20
			19/C/IP	*Conger conger*	70
			20/C/IP	*Conger conger*	30
			21/C/IP	*Conger conger*	60

Histological analysis carried out on liver samples of specimens caught in the Southern Adriatic Sea led to the following considerations:

- all specimens showed a liver struck by strong hyperaemia and degenerative phenomena;
- hepatocytes appeared with empty cytoplasm and reduced round eccentric nucleus;
- hepatic cellular membranes were often fragmented due to the presence of focal necrocitic phenomena;
- focal hepatitis was evident in some specimen as well as periportal fibrosis;
- hepatic parenchyma was full of granulomas;

- lipid degeneration was evident;
- histological analysis of the gills showed the presence of an inflammatory process with hyperaemia of the vascular apparatus;
- splenic tissues showed an increased number of melano-macrophagic centres.

Histological observations are summarised in table 5–19.

Tab. 5–19: histological lesions seen in tissues of specimens collected both in the pilot and in the reference area

Southern Adriatic				**Southern Tyrrenian**			
Specimen	Species	Histology		Specimen	Species	Histology	
		Liver	*Spleen*			Liver	*Spleen*
1/IP	H. dactylopterus	1,2,4,6	Normal	1/C/IP	H. dactylopterus	Normal	Normal
2/IP	*H. dactylopterus*	1,2,4,6	"	2/C/IP	*H. dactylopterus*	"	"
3/IP	*H. dactylopterus*	1,2,4,6	"	3/C/IP	*H. dactylopterus*	1	"
4/IP	*H. dactylopterus*	1,2,4,6	"	4/C/IP	*H. dactylopterus*	Normal	"
5/IP	*Trigla lira*	1	"	5/C/IP	*H. dactylopterus*	"	"
6/IP	*H. dactylopterus*	1,2,4,6	"	6/C/IP	*H. dactylopterus*	"	2
7/IP	*H. dactylopterus*	1,2,4,6	"	7/C/IP	*H. dactylopterus*	"	2
8/IP	*H. dactylopterus*	1,2,4,6	"	8/C/IP	*H. dactylopterus*	"	Normal
9/IP	*H. dactylopterus*	1,2,4,6	"	9/C/IP	*H. dactylopterus*	"	"
10/IP	*Conger conger*	2	2	10/C/IP	*H. dactylopterus*	"	"
11/IP	*Trigla lucerna*	1	Normal	11/C/IP	*H. dactylopterus*	"	2
12/IP	*Conger conger*	2,5	2	12/C/IP	*H. dactylopterus*	"	2
13/IP	*Raja asterias*	1	2				
14/IP	*Trigla lucerna*	Normal	Normal				
15/IP	*Trigla lucerna*	"	1				
16/IP	*Raja clavata*	1,4	Normal				
17/IP	Raja asterias	1,3	2				
18/IP	*Trigla lucerna*	Normal	Normal				

Legend

Liver: steatosis (1), fibrosis (2), glycogenosis, (3), focal hepatitis (4), granulomas (5), hyperaemia (6)
Spleen: atrophy of lymphatic centres (1), melano macrophages centres (2)

The analytical results indicate that leakage of CWAs from the rusted bombshells is likely to produce negative effects on the benthic ecosystem concerned.

The results of the A.C.A.B. project validated public interest on the matter and showed the need for further investigation. In fact, although the data seemed to confirm initial worries as regards both the extent and the ecological impact of this kind of pollution, the authors considered it necessary to stress that the results obtained were only preliminary and that further studies were needed. These results were taken into account when developing the RED COD project, which may become the follow up to the ACAB project, an important case study to refer to in similar, future works.

5.8 CWAs: sampling campaigns

5.8.1 Sampling campaign protocol

Specimen of *Helicolenus dactylopterus* (De La Roche) and *Conger conger* (L.), were collected preferentially by long lines. Following the studies carried out within the A.C.A.B. project these species have shown to be the most sensitive to the presence of chemical agents. The aim of the campaigns was to collect at least 20 specimens for each species per sampling site in order to obtain statistically significant results. Organs and tissues were taken from each specimen in order to carry out the following analyses, detailed in paragraph 6:

- Content of chemical warfare agents and their hydrolysis products;
- Concentrations of Hg and As (present in detonators and munitions and in many molecules used as CWAs, respectively);
- Histopathology;
- Stress indexes
- DNA damages

The analysed organs, conservation methods and the laboratories in charge of the analyses are summarised in the following table:

Tab. 5–20: Organs or tissues collected, conservation methods and analyses

Org./tissues	Analyses[126]	Storage methods[127]	Laboratories[128]
Blood	Ematic values (stress index)	N_2	Co.N.I.S.Ma. Siena
	Micronuclei (DNA damage)	blood slides	ICRAM
	CWAs concentration	-20 °C	CTLI NBC
Liver	MFO (stress index)	N_2	Co.N.I.S.Ma. Siena
	DNA Damage	N_2	Co.N.I.S.Ma. Bari
	CWAs content	-20 °C	CTLI NBC
	Hystopatology	Bouin/alchool 80°	ICRAM
Brain	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Gills	DNA Damage	N_2	Co.N.I.S.Ma. Bari
	CWAs content	-20 °C	CTLI NBC
	Hystopatology	Bouin/alchool 80°	ICRAM
	As and Hg content	-20 °C	Co.N.I.S.Ma. Siena
	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Muscle	DNA Damage	N_2	Co.N.I.S.Ma. Bari
	CWAs content	-20 °C	CTLI NBC
	As and Hg content	-20 °C	Co.N.I.S.Ma. Siena
	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Glad-bladder	CWAs content	-20 °C	CTLI NBC
Spleen	Hystopatology	Bouin/alcohol 80°	ICRAM
Gonads	Hystopatology	Bouin/alcohol 80°	ICRAM
	sex determination	Bouin/alchool 80°	ICRAM
	DNA Damage	N_2	Co.N.I.S.Ma. Bari
Kidney	Hystopatology	Bouin/alcohol 80°	ICRAM
	DNA Damage	N_2	Co.N.I.S.Ma. Bari
Gut	DNA Damage	N_2	Co.N.I.S.Ma. Bari
Skin	Hystopatology	Bouin/alcohol 80°	ICRAM
Otoliths	Age determination	Vials	ICRAM
Vertebrae	Age determination	Eppendorf	ICRAM

[126] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase; CWAs = Chemical Warfare Agents

[127] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

[128] Co.N.I.S.Ma. Siena = Co.N.I.S.Ma. – Local Unit of Siena University
Co.N.I.S.Ma. Bari = Co.N.I.S.Ma. – Local Unit of Bari University
CTLI NBC = *Centro Tecnico Logistico Interforze NBC - Civitavecchia* (Rome)

Field activities have been "standardised" in order to follow the same sampling procedure in the study areas and the reference sites, reducing the variables that might influence the final analytical result.

Specimens were collected by a sea bottom long line, being particularly careful to carry out comparable procedures during the different catches. This aspect has a certain importance particularly for the results of the stress indexes, as values can change in a proportional way to the time elapsed between the capture and the drawing up of the specimens. Furthermore it was properly checked that the fishing depth was comparable in all sampling sites in order to not consider this variable when evaluating the laboratory results.

While working on the field the animals collected were kept alive in tanks provided with oxygenators or with running water (fig. 5–63). Considering the high degradability of some molecules which we aimed to analyse (enzymes and DNA), it was our prime concern to reduce the period of time between the death of the specimen and the organ/tissue collection. In fact, it is very well known that only a few minutes after the collection, liver enzymes begin to degrade due to the action of a lysase, a hepatic enzyme. When the capture was abundant, the specimens most recently caught were chosen.

Fig. 5–63 Specimens of H. dactylopterus kept alive prior to tissues collection.

Fig. 5–64 tissue collection

Due to the high number of samples and their different ways of conservation, sampling campaigns had to be carried out with the presence of at least 4 operators. Specific data sheets, reporting the main information details for each specimen, were created (Annex VII).

After having measured the specimens (weight and length), plasma and serum aliquots, as well as samples of gall-bladder, muscle, liver and gill, they were stored at -20°C for further chemical analyses aimed at the detection of chemical

warfare agents and heavy metals tenors. Additional samples of plasma and serum, liver, muscle, gills and brain were stored in liquid nitrogen for biomarkers analyses together with another aliquot of gills, liver, gonads, kidney and gut for DNA damage analyses. Portions of skin, spleen, liver, gills and kidney were transferred in bouin solution and after 24 hours fixed in alcohol 80° for histological analyses. Five blood slides for each specimen were collected for micronuclei analyses.

Each specimen was accurately examined in order to detect pathological lesions. The following analyses were carried out:

- external inspection: evaluation of the dermis, gills, fins and eye status.
- internal inspection: observation of viscera *in situ*.

All samples have been placed in aluminium paper, except for the aliquot of blood placed on object slides and destined to the micronucleus test and for the sub samples destined to the analysis of heavy metals, which were preserved in PVC containers in order to avoid external metal contaminations.

In order to gather additional information on the collected specimens, otoliths (little calcareous concretions present in fish skull) and vertebrae were also collected and analysed. By observing a section of these tissues under a microscope, it is easy to discover the presence of "growth rings" showing variable tissue growth rate**.** If this variation has a defined temporal amplitude (a season, a month, a year), the number of rings can indicate the specimen's age [129] [130] [131] [132].

The otoliths have been hand-cleaned and dry-preserved in eppendorf. The vertebrae, taken at almost 3 cm from the anal pore, have been hand-cleaned and preserved in seawater in Falcon containers (Annex VIII).

[129] O'Sullivan S., Moriarty C., FitzGerald R. D., Davenport J., Mulcahy M. F., 2003. *Age, growth and reproductive status of the European conger eel, Conger conger (L.) in Irish coastal waters*. Fisheries Research, **64**: 55-69

[130] Pedersen J., 1997. *Comparison of vertebrae and otoliths measured directly and from radiographs.* Fisheries Research, **29**: 277-282.

[131] Colloca F., Cardinale M., Marcello A., Ardizzone G.D., 2003. *Tracing the life history of red gurnard (Aspitrigla cuculus) using validated otolith annual rings.* J. Appl. Ichthyol. **19**: 1-9.

[132] Svedang H., Wickstrom H., Reizenstein M., Holmgren K., Florenius P., 1998. *Accuracy and precision in eel age estimation, using otoliths of known and unknown age.* Journal of Fish Biology, **53**: 456-464.

Fig. 5–65 Detail of a Conger conger otolith

5.8.2 Southern Adriatic Sea

The sampling campaign aimed at the collection of specimens of *Conger conger* and *Helicolenus dactylopterus* in the chemical weapons dumping site in the Southern Adriatic Sea (fig. 5–67) was carried out during the period 17th - 20th July by the F/V "*Attila"* of Monopoli (Bari).

Fig. 5–66 Sailing of longline on board the F/V "*Attila"*

Luigi Alcaro, Marco Matiddi, Tommaso Petochi from ICRAM and Marzia Umani from CoNISMA participated in the sampling activities. The chosen fishing area is situated in the stretch of sea nearly 10 square nautical miles wide at about 35 miles from the coast, which had already been explored with electronic instruments during the A.C.A.B project (par. 5.7.1). In this area, reaching 200-300 m in depth, several chemical aerial bombs were detected and filmed. The vertex coordinates of the quadrilateral are shown hereafter:

A	φ 41°46'01"N λ 016°53'10"E	C	φ 41°45'56"N λ 016°53'17"E
B	φ 41°46'02"N λ 016°53'16"E	D	φ 41°45'56"N λ 016°53'10"E

A 1000 metres long sea bottom longline, with 500 hooks, was utilised to collect the organisms, using *Sardinia pilchardus* as bait. Nearly 3 hours after the placing, the longline was sailed. Samples were collected and stored as described in the previous paragraph.

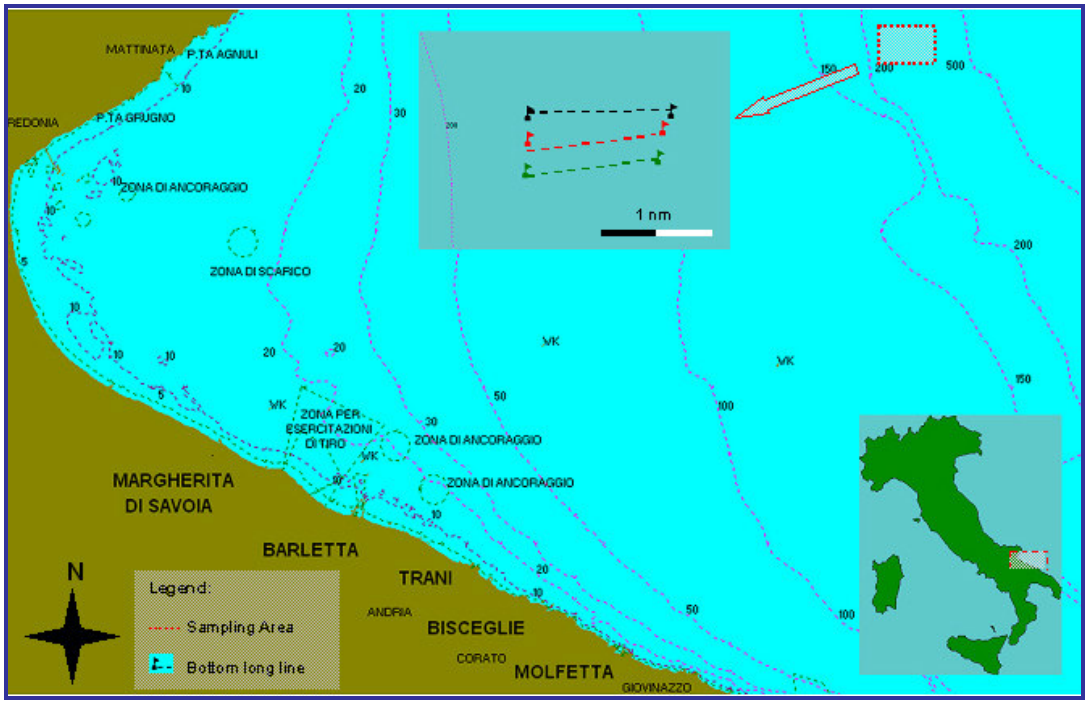


Fig. 5–67 Southern Adriatic Sea Sampling Area. July17th - 20th 2004

The main data concerning the sampled organisms is summarised in the following tables:

Table 5–21: Weight and length of the specimens of *H. dactylopterus* collected in the Southern Adriatic Sea

Specimen code	Weight (kg)	Total length (cm)	Specimen code	Weight (kg)	Total length (cm)
M0104H	n.d.	32	M1104H	600	31
M0204H	n.d.	35.5	M1204H	700	33
M0304H	n.d.	34.5	M1304H	450	28.5
M0404H	n.d.	18.5	M1404H	400	26.5
M0504H	n.d.	30	M1504H	500	27.5
M0604H	1.300	40	M1604H	400	29
M0704H	850	n.d.	M1704H	400	27.5
M0804H	750	34.5	M1804H	550	31.5
M0904H	700	33.5	M1904H	500	31
M1004H	850	35	M2004H	350	27
n	15	19			
Mean	533.4	31.1			
Mode	400	31			
Dev. St.	221.5	3.8			

Table 5–22: Weight and length of *C. conger* collected in the Southern Adriatic Sea

Specimen code	Weight (g)	Tot. length (cm)	Specimen code	Weight (g)	Tot.l length (cm)
M0104C	n.d.	84	M1104C	1,600	76.5
M0204C	n.d.	92	M1204C	1,700	98.5
M0304C	n.d.	144	M1304C	900	80
M0404C	n.d.	95	M1404C	1,100	83.5
M0504C	n.d.	97	M1504C	1,450	89
M0604C	n.d.	145	M1604C	3,000	110
M0704C	n.d.	91	M1704C	1,600	91.5
M0804C	1,200	87.5	M1804C	900	80
M0904C	n.d.	135	M1904C	800	73
M1004C	1,300	105	M2004C	700	70.5
n	12	20			
Mean	1354.2	96.4			
Mode	1600	80			
Dev. St.	617.7	21.8			

Table 5–23: Tissues of specimens of *H.dactylopterus* collected in Southern Adriatic Sea: analyses, storage methods and number of samples

Org./tissues	Analyses[133]	Storage methods[134]	No of samples
Blood	Ematic values (stress index)	N_2	36
	Micronuclei (DNA damage)	blood slides	100
	CWAs concentration	-20 °C	20
Liver	MFO (stress index)	N_2	20
	DNA Damage	N_2	20
	CWAs content	-20 °C	19
	Hystopatology	Bouin/alchool 80°	20
Brain	AChE (Stress index)	N_2	20
Gills	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	Hystopatology	Bouin/alchool 80°	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Muscle	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Glad-bladder	CWAs content	-20 °C	19
Spleen	Hystopatology	Bouin/alcohol 80°	20
Gonads	Hystopatology	Bouin/alcohol 80°	20
	sex determination	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Kidney	Hystopatology	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Gut	DNA Damage	N_2	20
Skin	Hystopatology	Bouin/alcohol 80°	20
Otoliths	Age determination	Vials	36
Vertebrae	Age determination	Eppendorf	-

[133] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase; CWAs = Chemical Warfare Agents

[134] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

Table 5–24: Tissues of specimens of *C. conger* collected in Southern Adriatic Sea: analyses, storage methods and number of samples

Org./tissues	Analyses[135]	Storage methods[136]	No of samples
Blood	Ematic values (stress index)	N_2	40
	Micronuclei (DNA damage)	blood slides	100
	CWAs concentration	-20 °C	20
Liver	MFO (stress index)	N_2	20
	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	Hystopatology	Bouin/alcohol 80°	20
Brain	AChE (Stress index)	N_2	20
Gills	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	Hystopatology	Bouin/alcohol 80°	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Muscle	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Glad-bladder	CWAs content	-20 °C	20
Spleen	Hystopatology	Bouin/alcohol 80°	20
Gonads	Hystopatology	Bouin/alcohol 80°	20
	sex determination	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Kidney	Hystopatology	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Gut	DNA Damage	N_2	20
Skin	Hystopatology	Bouin/alcohol 80°	20
Otoliths	Age determination	Vials	40
Vertebrae	Age determination	Eppendorf	20

[135] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase; CWAs = Chemical Warfare Agents

[136] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

5.8.3 Southern Tyrrhenian sea

Reference samples of *Conger conger* and *Helicolenus dactylopterus* were collected during the period 1st - 8th August in the Southern Tyrrhenian Sea (north of Sicily, in front of Capo d'Orlando) in an area not affected by the presence of dumped ordnance (fig. 5–69). The absence in the area of any kind of ordnance on the seabed was confirmed by an archive research and through interviews with fishermen. Sampling activities were carried out by: Camilla Della Torre, Pierpaolo Giordano, Tommaso Petochi from ICRAM, Michele Panza and Marzia Umani from CoNISMA.

Specimens were collected by long line fishing using *Sardina pilchardus* as bait.

Fig. 5–68 Sampling activities in front of Capo d'Orlando (reference area)

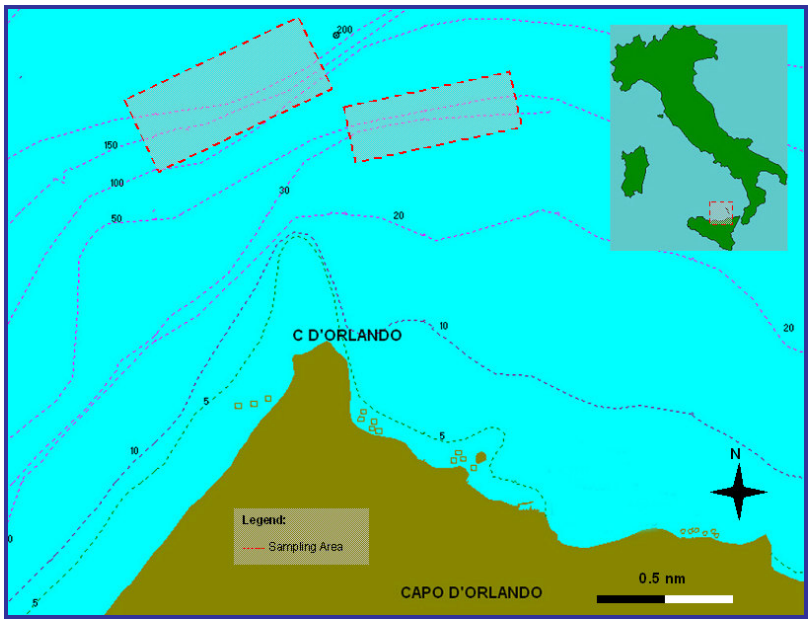


Fig. 5–69 Southern Tyrrhenian Sea Sampling Areas. August 1st - 8th 2004

All sampling procedures were comparable to those applied during the sampling campaign in the Southern Adriatic Sea.

The main data concerning the sampled organisms are summarised in the following tables:

Table 5–25: Weight and length of the specimens of *H. dactylopterus* collected in the Southern Tyrrhenian Sea

Specimen code	Weight (g)	Total length (cm)	Specimen code	Weight (g)	Total length (cm)
CD0104H	200	22	CD1104H	150	21.1
CD0204H	200	21	CD1204H	250	23.1
CD0304H	500	27	CD1304H	410	27
CD0404H	400	23	CD1404H	350	25.3
CD0504H	360	25	CD1504H	180	20.5
CD0604H	140	20.3	CD1604H	300	26
CD0704H	200	21	CD1704H	300	26
CD0804H	350	36.2	CD1804H	280	24
CD0904H	250	26	CD1904H	300	26
CD1004H	300	28	CD2004H	180	19
n	20	20			
Mean	280	24.4			
Mode	300	26			
Dev. St.	95.9	3.8			

Table 5–26: Weight and length of the specimens of *Conger conger* collected in the Southern Tyrrhenian Sea

Specimen code	Weight (g)	Total length (cm)
CD0104C	4,800	125
CD0204C	1,850	98
CD0304C	1,535	85
CD0404C	1,703	88
CD0504C	2,177	95.5
CD0604C*	n.d.	119.5
CD0704C*	n.d.	66
CD0804C*	n.d.	88
n	5	8
Mean	2,413	95.6
Mode	#N/D	88
Dev. St.	1355.1	19.1

*specimen collected by bow-nets.

Table 5–27: Tissues of the specimen of *H. dactylopterus* collected in the Southern Tyrrhenian Sea: analyses, storage methods and number of samples

Org./tissues	Analyses foreseen[137]	Storage methods[138]	no of samples
Blood	Ematic values (stress index)	N_2	38
	Micronuclei (DNA damage)	blood slides	100
	CWAs concentration	-20 °C	19
Liver	MFO (stress index)	N_2	20
	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	Hystopatology	Bouin/alcohol 80°	20
Brain	AChE (Stress index)	N_2	20
Gills	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	Hystopatology	Bouin/alcohol 80°	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Muscle	DNA Damage	N_2	20
	CWAs content	-20 °C	20
	As and Hg content	-20 °C	20
	AChE (Stress index)	N_2	20
Glad-bladder	CWAs content	-20 °C	19
Spleen	Hystopatology	Bouin/alcohol 80°	20
Gonads	Hystopatology	Bouin/alcohol 80°	20
	sex determination	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Kidney	Hystopatology	Bouin/alcohol 80°	20
	DNA Damage	N_2	20
Gut	DNA Damage	N_2	20
Skin	Hystopatology	Bouin/alcohol 80°	19
Otoliths	Age determination	Vials	40
Vertebrae	Age determination	Eppendorf	-

[137] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase; CWAs = Chemical Warfare Agents

[138] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

Table 5–28: Tissues of specimens of *C. conger* collected in Southern Tyrrhenian Sea: analyses, storage methods and number of samples

Org./tissues	Analyses foreseen[139]	Storage methods[140]	no of samples
Blood	Ematic values (stress index)	N_2	16
	Micronuclei (DNA damage)	blood slides	40
	CWAs concentration	-20 °C	8
Liver	MFO (stress index)	N_2	8
	DNA Damage	N_2	8
	CWAs content	-20 °C	8
	Hystopatology	Bouin/alcohol 80°	8
Brain	AChE (Stress index)	N_2	8
Gills	DNA Damage	N_2	8
	CWAs content	-20 °C	8
	Hystopatology	Bouin/alcohol 80°	8
	As and Hg content	-20 °C	8
	AChE (Stress index)	N_2	8
Muscle	DNA Damage	N_2	8
	CWAs content	-20 °C	8
	As and Hg content	-20 °C	8
	AChE (Stress index)	N_2	8
Glad-bladder	CWAs content	-20 °C	7
Spleen	Hystopatology	Bouin/alcohol 80°	8
Gonads	Hystopatology	Bouin/alcohol 80°	8
	sex determination	Bouin/alcohol 80°	8
	DNA Damage	N_2	8
Kidney	Hystopatology	Bouin/alcohol 80°	8
	DNA Damage	N_2	8
Gut	DNA Damage	N_2	8
Skin	Hystopatology	Bouin/alcohol 80°	8
Otoliths	Age determination	Vials	14
Vertebrae	Age determination	Eppendorf	6

[139] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase; CWAs = Chemical Warfare Agents

[140] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

5.9 TNT: sampling campaigns at the Tremiti Islands

5.9.1 Sampling campaign protocol

Sampling activities followed by an analytical protocol similar to the one developed for the campaign previously described. In this case two sampling surveys within the Protected Marine Area of the Tremiti archipelago (Southern Adriatic Sea) were planned: the first one in front of Pianosa Island, where many aerial bombs from WWII are present; the second campaign was carried out in front of the other islands of the archipelago, chosen as the reference site.

Specimen of *Conger conger* and *Paracentrotus lividus* (sea urchin) were collected for analysis. The aim was to evaluate any environmental effects due to polluting substances deriving from the corroded bombs (heavy metals, TNT and its degradation and hydrolysis products).

Concerning the sampling of conger eel, nearly 20 specimens were collected at each sampling site using sea bottom long lines.

Fig. 5–70 Weight measurement operation on board O/V Astrea

Fig. 5–71 Collection of blood sample from the caudal vein of a specimen of *C. Conger*, on board O/V Astrea

Organs and tissues were collected from each specimen in order to perform the following analyses:

- explosive compounds and their by-products contents

- concentrations of heavy metals
- stress indexes (alteration of mixed monoxygenase system in liver and esterase activity in muscle, gills and brain)
- DNA damage (micronucleus test)

As in the previous study, sampling activities required the presence of at least 4 operators. Samples of conger eel tissues and organs were collected with the same methodology utilised in the previous campaign and the main data was recorded on proper forms (annex VII).

The analyses, the conservation methods and the laboratories in charge of the analyses are summarised in the table below:

Tab. 5–29: Organs or tissues collected, conservation methods and analyses

Org./tissues	Analyses[141]	Storage methods[142]	Laboratories[143]
Blood	Ematic values (stress index)	N_2	Co.N.I.S.Ma. Siena
	Micronuclei (DNA damage)	blood slides	ICRAM
	TNT concentration	-20 °C	CTLI NBC
Liver	MFO (stress index)	N_2	Co.N.I.S.Ma. Siena
	TNT content	-20 °C	CTLI NBC
	Heavy metals content	-20 °C	Co.N.I.S.Ma. Siena
Brain	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Gills	TNT content	-20 °C	CTLI NBC
	Heavy metals content	-20 °C	Co.N.I.S.Ma. Siena
	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Muscle	TNT content	-20 °C	CTLI NBC
	Heavy metals content	-20 °C	Co.N.I.S.Ma. Siena
	AChE (Stress index)	N_2	Co.N.I.S.Ma. Siena
Gall-bladder	TNT content	-20 °C	CTLI NBC
Gonads	sex determination	Bouin/alcohol 80°	ICRAM
Otoliths	Age determination	Vials	ICRAM
Vertebrae	Age determination	Eppendorf	ICRAM

As for *Paracentrotus lividus* 84 specimen were collected in order to carry out a comparative analysis of stress markers (hsp70, metallothionein, AChE) expressed by coleomocytes (immuno-competent cells) of sea urchins between the pilot area and the control site.

Sea urchins were measured and the sex was determined. Coelomic fluid was collected from each specimen by cutting the peristomal membrane. Approximately 7.5 ml of the fluid was poured on the same volume of an anticoagulant solution ice-cold CCM2X: 1M NaCl - 10mM $MgCl_2$ - 40mMHepes [144]-2mMEGTA [145] pH7.2 [146]. The solution was centrifuged at 1000 rpm at 4°C for 5' and the pellet was

[141] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase;

[142] N_2 = Nitrogen liquid
Bouin/alchool 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

[143] Co.N.I.S.Ma. Siena = Co.N.I.S.Ma. – Local Unit of Siena University
Co.N.I.S.Ma. Bari = Co.N.I.S.Ma. – Local Unit of Bari University
CTLI NBC = Centro Tecnico Logistico Interforze NBC - Civitavecchia (Rome)

[144] 4-(2-Hydroxyethyl)piperazine-1-ethanesulphonic acid

[145] Ethylene glycol-bis(–βamminoethylether)-N,N,N',N'-tetracetic acid

[146] Henson, J. H., Nesbitt, D., Wright, B. D., Scholey, J. M., 1992. *Immunolocalization of Kinesin in sea urchin coelomocytes*. Journal of Cell Science 103: 309-320

stored in liquid nitrogen. Gonads were then collected and maintained at -20°C for chemical analyses (TNT and heavy metals content).

Fig. 5–72 *Paracentrotus lividus* Lamark, 1816 were sampled at the Tremiti Islands

Fig. 5–73 Preliminary analyses of *Paracentrotus lividus* on board the O/V "Astrea"

5.9.2 Pianosa Island

Sampling campaigns were carried out during the period June 20th July 5th 2004. Once the necessary permission was obtained from both the National Park of Gargano and the Harbour Office of Manfredonia, a temporary camp on the island was set up. The camp was necessary for logistic and sampling demands, considering the absence of inhabitable buildings. The environmental organisation "*Il mare accanto a noi*" provided the researchers with logistic aid.

Fig. 5-74 Camp organised using disused buildings

Ezio Amato, Luigi Alcaro, Camilla della Torre, Pierpaolo Giordano, Marco Matiddi, Valerio Sammarini, from ICRAM; Marzia Umani from CoNISMA; Annalisa Pinsino from IBIM participated in the sampling campaign. The activities included both underwater observations by means of divers and the collection of the specimens of *C. conger* and *P. lividus*

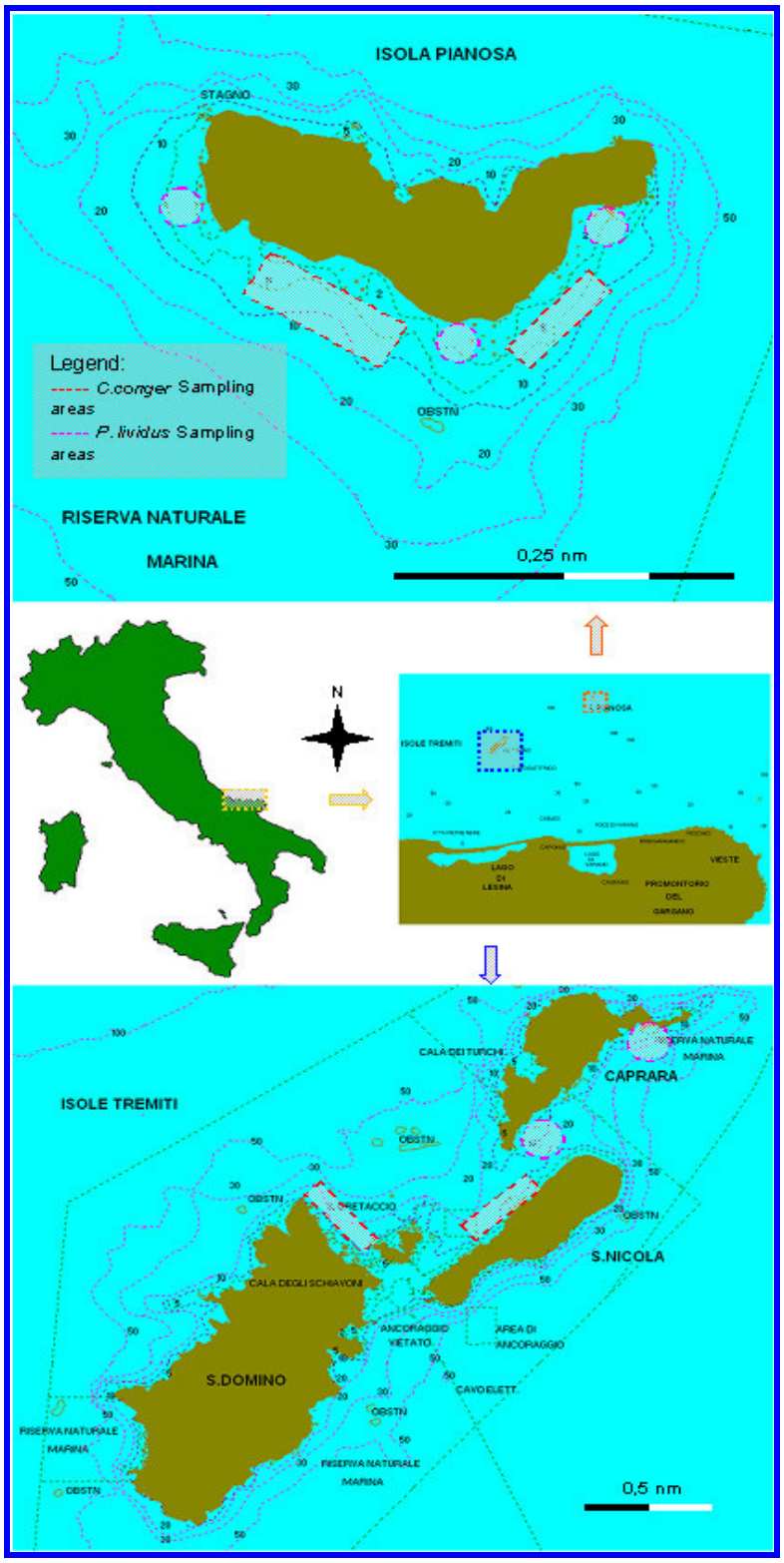


Fig. 5–73 Tremiti Sampling Areas. June 20th – July 5th 2004

The main data concerning the sampled organisms is reported in the following tables.

Table 5–30: Weight and length of specimens of *Conger conger* collected in Pianosa

Specimen	Weight (g)	Total length (cm)
P0104	2,200	99
P0204	1,200	79
P0304	1,500	93
P0404	600	69
P0504	750	71
P0604	2,500	108
P0704	850	76
P0804	1,180	85
P0904	3,100	110
P1004	500	44
P1104	1,250	90
P1204	2,000	90
P1304	1,650	89
P1404	1,350	86
P1504	350	55
P1604	600	58
P1704	400	67
P1804	2,200	106
P1904	200	51.5
P2004	200	47
P2104	300	54
P2204	950	78
P2304	1,000	71
n	23	23
Mean	1115.3	77.2
Mode	200	90
Dev. St.	835	19.7

Table 5–31: Tissues of specimens of *C.conger* collected in Pianosa: analyses, storage methods and number of samples

Org./tissues	Analyses[147]	Storage methods[148]	No. of samples
Blood	Ematic values (stress index)	N_2	20
	Micronuclei (DNA damage)	blood slides	115
	TNT concentration	-20 °C	20
Liver	MFO (stress index)	N_2	20
	TNT content	-20 °C	23
	Heavy metals content	-20 °C	23
Brain	AChE (Stress index)	N_2	20
Gills	TNT content	-20 °C	17
	Heavy metals content	-20 °C	22
	AChE (Stress index)	N_2	20
Muscle	TNT content	-20 °C	23
	Heavy metals content	-20 °C	23
	AChE (Stress index)	N_2	20
Glad-bladder	TNT content	-20 °C	20
Gonads	sex determination	Bouin/alcohol 80°	23
Otoliths	Age determination	Vials	44
Vertebrae	Age determination	Eppendorf	22

[147] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase;

[148] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

Table 5–32: Weight and length of specimens of *Paracentrotus lividus* collected in Pianosa

Specimen code	Sex	Size (cm)
PE1	F	5
PE2	F	4.5
PE3	F	5
PE4	F	5
PE5	M	3.5
PE6	F	5.3
PE7	M	4.8
PE8	M	4.9
PE9	F	4.1
PE10	F	4.4
PE11	F	4.9
PE12	F	4.8
PE13	F	5.1
PE14	M	5
PE15	M	5
PE16	F	4.7
PE17	M	5
PE18	F	4
PE19	M	5.6
PE20	M	4.9

Specimen code	Sex	Size (cm)
PF1	F	3.9
PF2	F	4.9
PF3	M	5.3
PF4	F	5.5
PF5	F	4.7
PF6	F	4.7
PF7	F	5.4
PF8	F	4.9
PF9	F	3.8
PF10	F	5
PDM	F	4.6
PD1	F	4.1
PD2	F	5.2
PD3	F	4.4
PD4	M	4
PD5	M	4
PD6	F	4.4
PD7	M	4.7
PD8	F	5.5
PD9	F	5.6

Specimen code	Sex	Size (cm)
PD10	F	5.4
PD11	M	5.6
PD12	M	5.9
PD13	F	4.9
PD14	M	4.8
PD15	F	4.6
PD16	F	5.4
PD17	M	4.7
PD18	M	5.5
PD19	F	5.5
PD20	F	5.6
PG1	F	4.8
PG2	M	5
PG3	F	4.7
PG4	F	5.3
PG5	F	4.8
PG6	F	5
PG7	M	4.7
PG8	F	4.9
PG9	M	4.4
PG10	F	4

n	61
Mean	4.8
Mode	4.7
Dev. St.	0.5

Table 5–33: Tissues of specimens of *P. lividus* collected in Pianosa: analyses, storage methods and number of samples

Org./tissues	**Analyses**[149]	**Storage methods**[150]	**No. of samples**
Gonads	TNT and heavy metals content	-20 °C	61
Coelomocytes	stress markers (hsp70, metallothionein, AChE)	N_2	61

[149] Hsp70 = Heat Shock Protein; AChE = Acetyl cholinesterase;

[150] N_2 = Nitrogen liquid

5.9.2.1 ORDNANCE OBSERVED ON THE SEAFLOOR

The analysis of the log books of several ships of the Italian Navy concerning the first years after the end of WWII clearly indicate Pianosa island as a military area[151].

In addition to the sampling of marine organisms, the research group carried out several underwater investigations around Pianosa island by scuba divers and three underwater vehicles (TEKNA DV3X and DACOR models) which facilitated the detection and observation of an important amount of war surplus.

Fig. 5–74 survey of the seafloor by underwater vehicles

On the basis of the information provided by the General Staff of the Italian Navy the numerous underwater aerial bombs observed have been classified as model DEMO of 100/125/250/500 pounds.

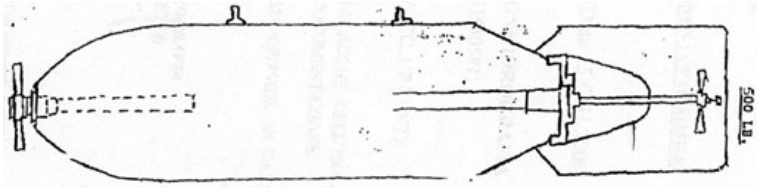


Fig. 5–75 Drawing of an aerial bomb "Demo" of 500 pounds

Most of the weapons appeared to be broken and/or corroded and without vane. In one case the bomb was integral but distorted, probably due to the impact with the

[151] State Archives of Italian Navy in Rome – Archivio LI. *Periodo Postbellico dopo l'8 maggio 1945*.

seafloor. Other bombs seemed to be integral and their surface was only partially colonised.

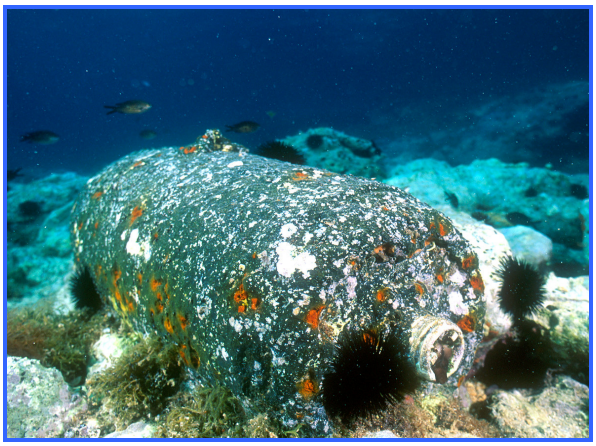


Fig. 5–76Conventional aerial bomb located on the seafloor off Pianosa Island with *Paracentrotus lividus*

Fig. 5–77 Another conventional aerial bomb located on the seafloor off Pianosa Island

Fig. 5–78 Conventional aerial bomb located on the seafloor off Pianosa Island.

The munitions were classified on the basis of their state of conservation, whereas their position was assessed taking into account bathymetry and observation of remarkable points (Fig. 5–79).

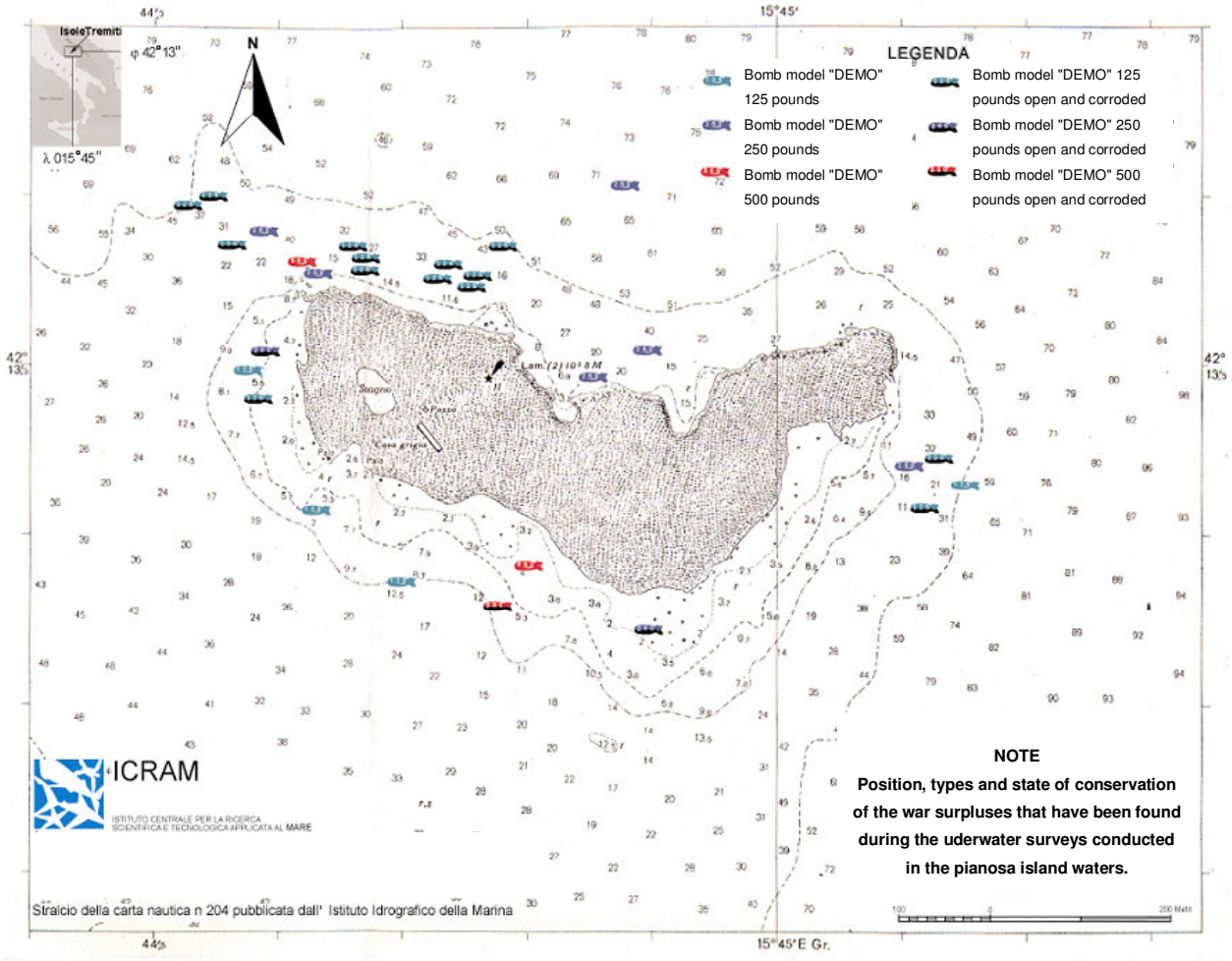


Fig. 5–79 Conventional weapons located on the seafloor off Pianosa Island and their state of corrosion

5.9.3 San Domino and Caprara Islands

All sampling campaigns were carried out during the same period. The reference organisms were collected by the same personnel present on Pianosa island by ICRAM's oceanographic vessel O/V “ASTREA”. (figg. from 5–80 to 5–82).

Fig. 5–80. Conservation of gonads collected from *Conger conger* specimen, on board the O/V "Astrea"

Fig. 5–81 Preliminary analysis of *Paracentrotus lividus* on board the O/V "Astrea"

Fig. 5–82 Gonad collection from *Conger conger* specimen on board the O/V "Astrea"

The main data concerning the organisms sampled in the reference area are summarised in the following tables:

Table 5–34: Weight and length of specimens of *Conger conger* collected in the reference area

Specimen code	Weight (g)	Total length (cm)	Specimen code	Weight (g)	Total length (cm)
TB0104	800	75	TB 1004	1,150	86
TB 0204	n.d.	84	TB 1104	400	53
TB 0304	n.d.	56	TB 1204	500	65
TB 0404	n.d.	60	TB 1304	1,000	79
TB 0504	n.d.	80	TB 1404	450	56
TB 0604	n.d.	70	TB 1504	1,100	84
TB 0704	n.d.	62	TB 1604	500	62
TB 0804	1,000	78	TB 1704	200	53
TB 0904	900	75	TB 1804	950	79
n	12	18			
Mean	745.8	69.8			
Mode	1,000	75			
Dev. St.	318	11.5			

Table 5–35: Tissues of specimens of *C.conger* collected in the reference area: analyses, storage methods and number of samples

Org./tissues	Analyses[152]	Storage methods[153]	No. of samples
Blood	Ematic values (stress index)	N_2	26
	Micronuclei (DNA damage)	blood slides	65
	TNT concentration	-20 °C	26
Liver	MFO (stress index)	N_2	18
	Heavy metals content	-20 °C	18
Brain	AChE (Stress index)	N_2	18
Gills	Heavy metals content	-20 °C	13
	AChE (Stress index)	N_2	18
Muscle	Heavy metals content	-20 °C	18
	AChE (Stress index)	N_2	18
Glad-bladder	TNT content	-20 °C	16
Gonads	sex determination	Bouin/alcohol 80°	54
Otoliths	Age determination	Vials	36
Vertebrae	Age determination	Eppendorf	18

[152] MFO = MultiFunction Oxigenase; AChE = Acetyl cholinesterase;

[153] N_2 = Nitrogen liquid
Bouin/alcohol 80° = the sample is fixed in Bouin for 24/48 h and then transferred in alcohol 80°

Table 5–36: Weight and length of the specimens of *Paracentrotus lividus* collected in the reference area

Specimen code	Sex	Size (cm)
TB1	M	4.5
TB2	M	4.5
TB3	M	4.4
TB4	M	4.3
TB5	F	3.9
TB6	M	4.7
TB7	M	4.5
TB8	M	4.9
TB9	F	3.9
TB10	M	4.8
TB11	F	5.0
TB12	F	5.7
TB13	M	4.9
TB14	F	4.8
TB15	F	4.6
TB16	F	4.4
TB17	F	4.7
TB18	M	4.7
TB19	F	3.9
TB20	F	4.7
TB21	M	5.3
TB22	M	4.2
TB23	M	5.3
n		23
Mean		4.6
Mode		4.7
Dev. St.		0.4

Table 5–37: Tissues of the specimens of *P. lividus* collected in the reference area: analyses, storage methods and number of samples

Org./tissues	**Analyses**[154]	**Storage methods**[155]	**No. of samples**
Gonads	TNT and heavy metals content	-20 °C	23
Coelomocytes	stress markers (hsp70, metallothionein, AChE)	N_2	23

[154] Hsp70 = Heat Shock Protein; AChE = Acetyl cholinesterase;

[155] N_2 = Nitrogen liquid

6 Laboratory Analyses Programme

On the basis of the RED COD proposal, the participants have carried out several laboratory analyses aimed at observing any potential damage to the benthic environment within both CWs and conventional dumping areas. This multidisciplinary approach involved analyses at (bio)chemical, cellular, tissue and organism level. Particularly, tissues contamination by yperite and by 2,4,6, trinitrotoluene (TNT) and theirs degradation products was investigated as well as concentrations of mercury (Hg) and arsenic (As) were considered. Skin, gills, liver, spleen, kidney and gonads of the fish were analysed for histopathology and stress indexes, DNA damage and the Heat Shock Protein (Hsp70) were investigated as well.

As the bibliographical research carried out so far highlighted a general lack of studies regarding the fate and the effects on the biota of the investigated substances once entered into the marine environment, the partners have agreed to perform two additional *in vivo* experiments in order to better understand the target organs of the pollutants (TNT and yperite), their chemical form once entered the organism and the most proper way to detect their effects on the biota.

During the *in vivo* experiments specimens of *Anguilla anguilla* (European eel) have been exposed to different concentrations of TNT and yperite under controlled laboratory conditions. It was agreed to choose *European eel* as the test organism since it is widely used in this kind of studies and its ecological characteristics are quite similar to the ones of the conger eel (*C. conger*) which was our target species. Furthermore *A. anguilla* has proved to be much more resistant than *C. conger* to aquarium living conditions. Its suitability to laboratory condition and handling[156] and its low sensitivity to xenobiotic exposure make this species a suitable model for toxicological studies[157]. The way and the period of exposure as well as the pollutants concentrations have been determined also on the basis of other similar studies performed on different species[158 159 160 161 162].

[156] Hewitt S., Fenet H. and Casellas C., 1998. Induction of EROD activity in European eel (*Anguilla anguilla*) by different polycholobiphenils (PCBs). Wat. Sci. Technol. **38**: 245-252.

[157] Regoli F., Winston G.W., Gorbi S., Frenzilli G., Nigro M., Corsi I., Focardi S., 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. Environ. Toxicol. Chem. **22** (9) :2120-9.

[158] Ownby D.R., Belden J.B., Lotufo G.R., Lydy M.J., 2005. Accumulation of trinitrotoluene (TNT in aquatic organisms: Part 1 – bioconcentration and distribution in channel catfish (*Ictalurus punctatus*). Chemosphere **58**: 1153-1159.

[159] Smock L.A., Stonuburner D.L., Clark J.R., 1976. The toxic effects of trinitrotoluene (TNT) and its primary degradation products on two species of algae and the fathead minnow. Water Research, **10**: 537-543.

The *Centro Tecnico Logistico Interforze NBC* located in Civitavecchia (Rome) and ICRAM have carried out the chemical analyses aimed at detecting the presence of yperite, TNT and their degradation products within samples collected during the above mentioned campaigns and those deriving from the *in vivo* studies.

The Co.N.I.S.Ma. (Local Unit of Siena University) has performed the mercury and arsenic concentration analyses on tissue samples collected during CWs campaigns. The detected mercury is supposed to derive from mercury fulminate ($(CNO)_2Hg$) used as detonator in the bombs whereas the arsenic is the characteristic atom included in some blistering agents like lewisite and other arsenic compounds (e.g. adamsite, phenyl dichloro arsine).

Moreover, this laboratory has carried out the stress indexes analyses on biological samples collected both in CWs and TNT sites as well as on samples deriving from the *in vivo* experiments. The analyses were mainly focused on the alteration of some enzyme activities (e.g. cytochrome P450 system and Cholinesterase (ChE)). Enzymatic activities may be altered by the presence of xenobiotic substances within the organism. The cytochrome P450 system is the most important multienzymatic complex involved in the xenobiotics detoxification process performed by the organism. In vertebrates, it is located on the membranes of the endoplasmic smooth reticulum, mostly in the liver[163]. ChE, instead, is an enzyme responsible for acetylcholine hydrolysis within the synaptic space thus being a specific marker for neurotoxic compounds[164].

The ICRAM laboratory has been in charge of developing the histopathological analyses of tissue samples collected during the CWs campaigns and the relevant *in vivo* experiment. The analyses were focused on specimens showing evident skin and internal tissues damages which are usually present in case of blistering chemical agents contamination (blister, necrosis, etc.).

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[160] Ek H., Goran D., Birgersson G. and Forlin L., 2003. Acute effects of 2,4,6-trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout (*Oncorhynchus mykiss)* Aquatic Ecosyst. Health Man., **6** (4): 415-421.

[161] Goldman M. and Dacre J.C., 1989. Lewisite: it's chemistry, toxicology and biological effects. In Reviews of environmental contamination and toxicology. Springer Verlag New York **110**: 75-115.

[162] Henriksson J., Johannisson A., Bergqvist P-A., Norrgren L., 1996. The toxicity of organoarsenic-based warfare agents: in vitro and in vivo studies. Arch. Environ. Contam. Toxicol. **30**: 213-219.

[163] GoksØyr A., Förlin L., 1992. The cytocrome P-450 system in fish, aquatic toxicology and enviromental monitoring. Aquatic Toxicology **22**: 287-312.

[164] Sturm A., da Silva de Assis H.C., Hansen P.D., 1999. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Mar. Env. Res. **47**: 389-398.

The Co.N.I.S.Ma. (local Unit of Bari University) has performed the DNA damage analyses in samples of specimens collected during CWs campaigns. The damage to the genome is one of the best-known effects of yperite. Mustard gas is a highly reactive bifunctional alkylating agent that forms sulfonium ions in the body. It alkylates DNA, leading to DNA strand breaks and cell death in a variety of cell types and tissues[165]. Several methods have been developed in order to detect DNA strand damage in freshwater and marine animals after *in vivo* and *in vitro* exposure to a variety of genotoxicants[166]. The Co.N.I.S.Ma. has utilized the Comet Assay technique for assessing DNA damage in cells isolated from different tissues (kidney, liver, muscle, gills and intestine).

Another method aimed at detecting DNA damages is the *Micronuclei* Test, which has been performed by ICRAM. *Micronuclei* are composed of small chromatin fragments which arise as a result of chromosome breaks after clastogenic action or due to the incomplete migration of chromosomes during the cell division (mitosis) as a result of aneugenic effects.

The IBIM-CNR has analysed the heat shock protein Hsp70 expression in coelomocytes of specimens of sea urchin *Paracentrotus lividus* (Lamark, 1916) collected during the TNT sampling campaigns. Coelomocytes have the capability to respond to cytotoxic agents as an immune system. In fact, coelomocytes are able to react to stress factors through modifications in their motility as well as in their phagocytic and encapsulation activities[167] [168] [169] [170] [171]. In particular, the coelomocytes are able to respond to environmental pollution by the up regulation of the Hsp70 gene and the accumulation of the corresponding protein that is enhanced when a stress factor occurs. Due to their physiological and

[165] Bhattacharya R., Lakshmana R., Pant S.C., Kumar O., Tulsawani R.K., Pathak U., Kulkarni A., Vijayaraghavan R., 2001. Protective effects of amifostine and its analogues on sulfur mustard toxicity in vitro and in vivo. Toxicol Appl. Pharmacol. **176**: 24-33.

[166] Lee R.F., Steinert S., 2003. Use of the single cell gel electrophoresis/comet assay for detecting DANN damage in aquatic (marine and freshwater) animals. Mutat. Res. **544**: 43-64.

[167] Matranga V., 1996. Molecular aspects of immune reactions in Echinodermata. Prog Mol Subcell Biol **15**: 235-247.

[168] Matranga V., Bonaventura R., 2002a. Sea urchin coelomocytes, the progenitors of vertebrates immune effectors, as bio-indicators of stress and pollution. In The Sea Urchin: from basic biology to aquaculture. Yokota Y, Matranga V & Smolenicka Z, eds (Swets & Zeitlinger, Lisse, The Netherlands): pp 161-176.

[169] Matranga V., Bonaventura R, Di Bella G., 2002b. Hsp70 as a stress marker of sea urchin coelomocytes in short term cultures. Cellular and Molecular Biology **48** (4): 345-349.

[170] Morale A., Coniglio L., Angelini C., Cimoli G., Bolla A., Alleteo D., Russo P., Falugi C., 1998. Biological effects of a neurotoxic pesticide at low concentrations on sea urchin early development. A terathogenic assay. Chemosphere **37**: 3001-3010.

[171] Warnau M., Biondo R., Temara A., Boquegneau J-M., Jangoux M., Dubois P., 1998. Distribution of heavy metals in the echinoid *Paracentrotus lividus* from the Mediterranean *Posidonia oceanica* ecosystem: seasonal and geographical variations. J Sea Res **39**: 267-280.

cytoprotective functions, Hsp70 proteins are able to develop a tolerance to a great variety of stress factors, enhancing cellular survival[172].

Sea urchin coelomocytes are now assumed to be ubiquitous biomarkers of exposure to environmental stress such as heavy metals[173] [174], temperature variation[173], UV radiation[175], organic contaminants[173] and oxidative stress[176] [177] [178].

6.1 CWAs: laboratory analyses

6.1.1 *In Vivo* Test

At present no data are available regarding mustard gas toxicity mechanism and the biochemical alterations occurring in aquatic fish species after exposure to this specific compound. In order to overwhelm this gap an *in vivo* experiment was performed by exposing specimens of European eel *Anguilla anguilla*, under controlled laboratory conditions, to different concentrations of mustard gas for different periods of time.

In particular, sixty specimens of European eel, with a mean weight of 60 g and a total length of 35 cm, were collected from the Orbetello lagoon (Tuscany, North Western Mediterranean Sea). Eels were acclimatized for 24 h in six 40 l aerated aquariums containing artificial sea water at 30‰ salinity and 18°C temperature.

[172] Samali A., Orrenius S., 1998. Heat shock proteins: regulators of stress response and apoptosis**.** Cell Stress Chaperones. **3**(4):228-36.

[173] Sanders B.M., Hope C., Pascoe V. and Martin L.S., 1991. Characterization of the stress protein response in two species of *Colisella limpets* with different temperature tolerances. Physiol Zool., **64**: 1471-1489.

[174] Bauman J.W., Liu J., Klaassen C.D., 1993. Production of metallothionein and heat-shock proteins in response to metals. Fundam. Appl. Tox. **21**(1):15-22.

[175] Nepple B.B. and Bachofen R., 1997. Induction of stress proteins in the phototrophic bacterium *Rhodobacter sphaeroides*. FEMS Microbiol. Lett., **153**: 173-180.

[176] Su C., Chong K., Edelstein K., Lille S., Khardori R. and Lai C., 1999. Costitutive Hsp70 attenuates hidrogenoperoxide-induced membrane lipid peroxidation. Biochem. Biophys. Res. Com., **265**: 279-284.

[177] Calabrese V., Scapagnini G., Catalano C., Bates T.E., Geraci D., Pennini G., Giuffrida S., 2001. Regulation of heat shock protein synthesis in human skin fibroblasts in response to oxidative stress: role of vitamin E. Int. J. Tissue. React., **23**(4):127-35.

[178] Menoret A., Chaillot D., Callahan M., Jacquin C., 2002. Hsp70, an immunological actor playing with the intracellular self under oxidative stress. Int J Hyperthermia, **18**(6):490-505.

Fig. 6–1 European eels in aquarium prior to the experiment. (Ph. Tommaso Petochi, ICRAM)

European eels were contaminated with yperite through an intramuscular injection following a slight anaesthesia with MS222 (Sigma) at a concentration of 200 mg/l.

Fig. 6–2 Injection of a solution of yperite in corn oil in a specimen of European eel. (Ph. Tommaso Petochi, ICRAM)

The operators have taken into account all safety procedures and equipment in order to minimize the risk of contamination.

Fig. 6–3 protective equipment utilised for injection operation. (Ph. Tommaso Petochi, ICRAM)

Fishes were divided into six groups of 10 specimens each:

- one group was injected with 100 µl of corn oil (Sigma Aldrich) and was considered as the reference;
- other four groups were injected with 100 µl of mustard gas dissolved in corn oil at different concentrations: 10 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml;
- one group was kept untreated in order to reveal potential effects due to corn oil or handling conditions.

Five fishes of each group were sacrificed after 24 and 48 h through beheading. Each specimen was subjected to autoptical analysis, measured and weighted. Tissues were collected for the following analyses: content of yperite and its degradation products, stress indexes and histopathology.

Blood was sampled from the caudal vein by a 2.5 ml heparinyzed syringe and it was divided into two aliquots, one for chemical analysis stored in heparinysed tubes and one for blood stress parameters which was centrifuged at 5000 rpm for 10 min. Samples of liver, gall bladder and muscle were collected and stored in liquid nitrogen and at -20°C for stress indexes evaluation and mustard gas content respectively. A portion of spleen, liver, skin, kidney and gills was transferred in Bouin solution and fixed in alcohol 80% for histopathological analysis. One specimen of the highest concentration group was stored intact at -20°C for further inspections.

6.1.2 Yperite and its Degradation Products in Fish Tissues

6.1.2.1 MATERIALS AND METHODS

The analysis of yperite and its degradation products within the tissues of the sampled organisms has been carried out following the procedure described in Drasch *et al.*[179]. As the protocol has been further modified and adapted to the specific case, a certain period of time has been dedicated to the development of the analytical procedure. In particular the choice of the reagents, the volumes involved as well as the recovery rate for yperite have been tested and evaluated.

6.1.2.1.1 Preliminary tests

The protocol concerned the following steps:

- extraction with dichloromethane (DCM);
- sample purification through silica cartridge;
- sample concentration prior to injection in GC/MS.

In order to test the methodology a series of experiments have been carried out. The first step was to verify whether yperite was able to pass through the silica capillary maintained in a cartridge without being retained and the volumes of solvent necessary to let this happen.

A solution of yperite 1.27 ppm and DCM was prepared while the silica capillary was conditioned prior to its first use by flushing with 12 ml of DCM; 1 ml of the above mentioned solution was injected into the capillary followed by 8 aliquots of 6 ml of solvent (48 ml in total). Each aliquot was concentrated to 1 ml and

[179] Drasch G., Kretschmer E., Kauert G., Von Meyer L., 1987. Concentrations of mustard gas [bis(2-chloroethyl)sulphide)] in the tissues of a victim of a vesicant exposure. J. For. Sci. **32** (6): 1788-1793.

analysed with a gas chromatograph/mass spectrograph. Finally methanol, being more polar than DCM, was passed through the capillary in order to verify that no more yperite was retained by the silica. Results confirmed that yperite is detected only within the first aliquot of 6 ml which means that it passes immediately through the cartridge without being retained.

The following step was to repeat the whole procedure by contaminating a sample of muscle and liver of *Anguilla anguilla* and extracting with 3 ml of DCM in an ultrasonic bath for 15 minutes. The sample was injected into the silica capillary, concentrated to the volume of 1 ml and analysed through a GC/MS. Results clearly demonstrated that the cartridge is able to retain fatty acids, thus rendering possible the analyses with GC, while yperite was significantly detected.

6.1.2.1.2 Samples preparation and analyses

Once the methodology was properly tested it was applied to samples of European eel contaminated *in vivo*. Samples of muscle and liver of *Anguilla anguilla*, both lyophilized and homogenized, were added to 3 ml of dichloromethane (DCM) and put in an ultrasonic bath for 15 minutes. The supernatant was collected and eluted through a STEPBIO silica capillary maintained in a cartridge in order to purify samples without retaining yperite or its by-products. The procedure was repeated three times and the final solution was concentrated to 1 ml at room temperature in order to be analysed through GC-MS.

Mustard gas standard (>90.0%) was obtained by the distillation of a mixture Y-FDA taken from the CeTLI demilitarization plants. The standard provided a good instrumental response and allowed to make the calibration curve preparing four solutions, whose concentration was respectively 0.5, 5, 10, 20 mg/L. Mass spectrometry detector response shows a linear correlation between concentration and signal intensity (Fig 6–4.).

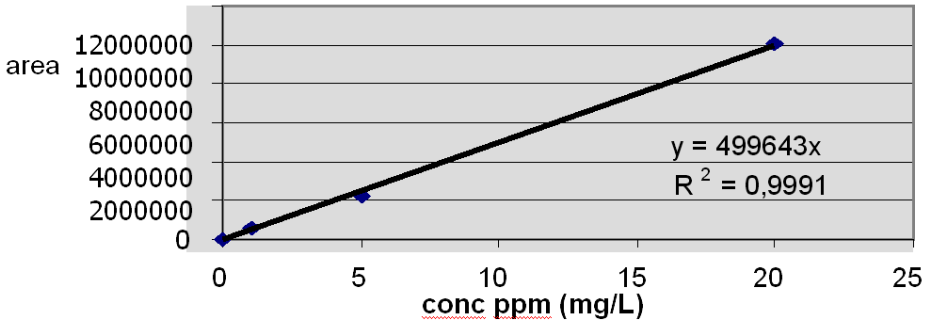


Table 6-4 Yperite calibration curve in DCM

Vials containing samples were put on the automatic system for auto sampling TRIplus Duo for GC-MS analysis. “GC TRACE” instrumental apparatus, including “POLARIS Q” ion-trap spectrometer is run by a special software called “Xcalibur”. Instrumental operating conditions are reported as follows:

GC Parameters

PTV (programmable temperature value) injector and melted silica capillary column Zebron-1MS (5mGuard) of 30 m; d.i.=0.25 mm; f=0.25 µm; operating parameters are as follows:

PTV, splitless mode, 50°C, 0.05min; + 14.5°C/s, 240°C, 300s; t_{purge}=0s, t_{split}=120s, Φ_{purge}=5 ml/min, Φ_{split}=50 ml/min; $T_{interface}$=250°C; carrier= He @ 0.8 ml/min (constant);

oven temperature: T_1=40°C, 3 min; +20°C/min; T_2=250°C, 2 min;

MS Parameters

electron energy= 70 eV, 33≤m/z ≤ 400, scan time= 0.44s, microscan=3; maximum ion time=25ms; AGC target=50; solvent delay 1.40 min. Scan mode: full scan (range 30-650) Sim mode: ion target HD (109,158,111).

6.1.2.2 RESULTS

The limit of detection for yperite has been experimentally determined as 0.1 ÷ 0.05 mg/L in the final solution, considering an injected volume of 1 µl in PTV mode or in splitless mode. Detectors used were either quadrupole or ion-trap, the latter being more accurate.

Chromatographic peak retention times have been examined on chromatograms obtained with ion-trap and quadrupole detectors in order to eventually reveal peaks of yperite and its degradation products. There is no evidence of compounds of interest in fish tissue samples (Fig. 6–5 and 6–6).

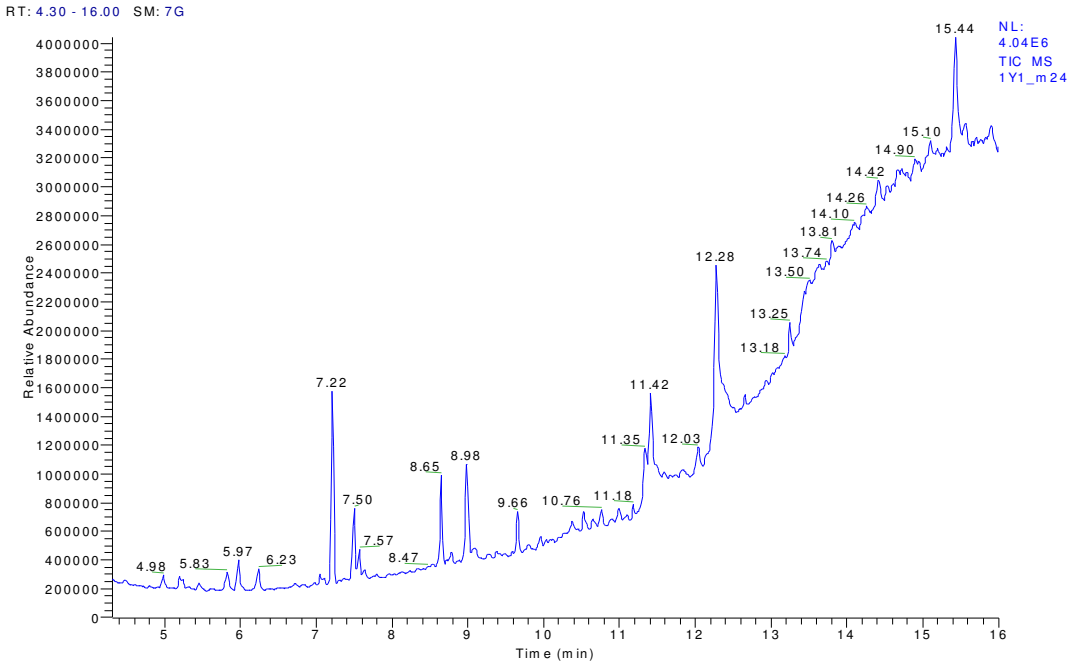


Fig. 6-5: muscle sample chromatogram of an *Anguilla anguilla* specimen treated with an injection of 1 mg/ml of yperite and sacrificed after 24 hours

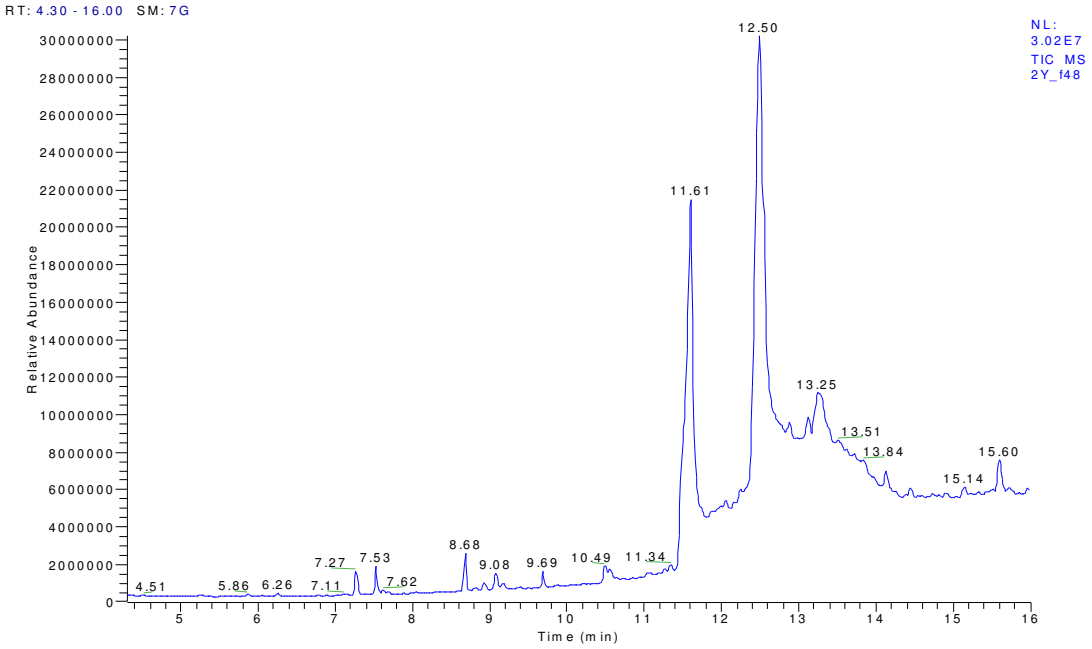


Fig. 6-6: liver sample chromatogram of an *Anguilla anguilla* specimen treated with an injection of 10µg/ml of yperite and sacrificed after 48 hours

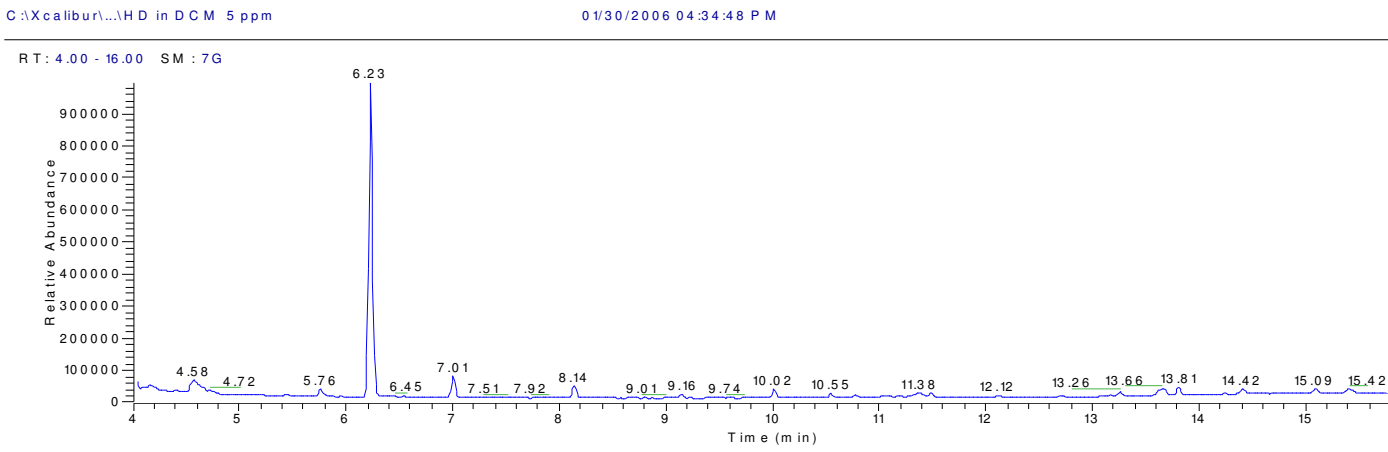
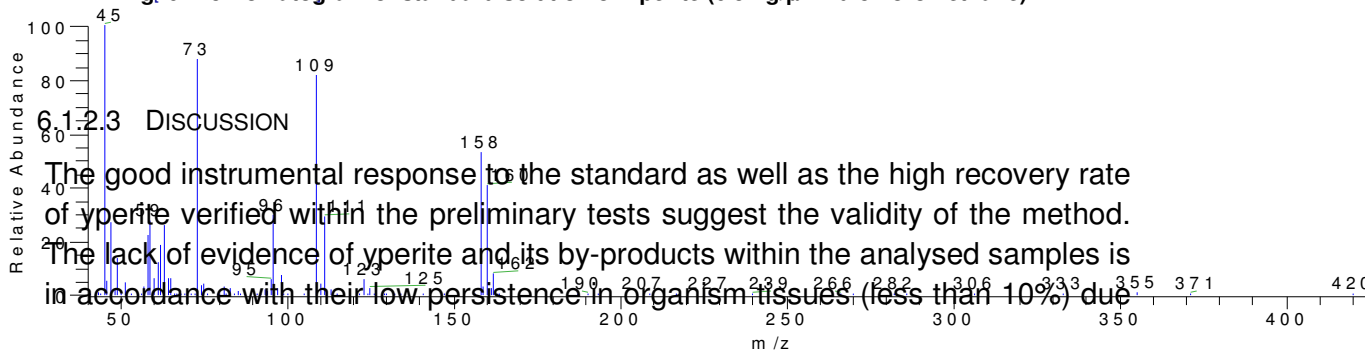


Fig. 6-7 chromatogram of standard solution of Yperite (0.5 ng/µl in dichloromethane)



6.1.2.3 DISCUSSION

The good instrumental response to the standard as well as the high recovery rate of yperite verified within the preliminary tests suggest the validity of the method. The lack of evidence of yperite and its by-products within the analysed samples is in accordance with their low persistence in organism tissues (less than 10%) due

to the rapid passage into circulation (blood) and their high reaction with proteins[180] [181] [182]. Thus, the absence of detectable traces of CWAs and their biodegradation products in fish tissues confirms their low bioaccumulation and bioconcentration capabilities[183].

6.1.3 Arsenic and Mercury in Fish Tissues

As already mentioned before, Arsenic (As) and Mercury (Hg) analyses in fish tissues were performed as these are among the main trace elements that could be released by rusted chemical weapons. The analyses were carried out in samples of muscle being the edible part of the organism and thus able to provide some indications on the potential risk for the consumers.

6.1.3.1 MATERIALS AND METHODS

Nearly 0.3 g of liophylized muscle tissue were decomposed in nitric acid and hydrogen peroxide and in a microwave unite (MILESTONE ETHOS 900). Mercury concentration in the final solution was determined with the CV-AAS by the Flow Injection Mercury System (Perkin Elmer FIMS 400). Arsenic was measured by using the ICP-OES (Perkin Elmer 5300 DV).

Dogfish reference material (DORM 2) from the National Research Council Canada (NRCC) was used as reference for analytical methods; quantitative agreement with the respective certified values was achieved for both elements.

6.1.3.2 RESULTS

Hg and As tenors determined in *H. dactylopterus* and *C. conger* muscle tissue are summarized in tables and figures. Hg and As levels measured in *H. dactylopterus* and *C. conger* from the CWAs-impacted site are significantly higher than those from the reference site, with three-four times ratio each.

[180] Collumbine H., 1947. Medical aspects of mustard gas poisoning. Nature **4031**: 151-153.

[181] Somani S.M., Babu S.R., 1989. Toxicodynamics of sulfur mustard. Int. J. Clin. Pharmacol. Ther. Toxicol., **27**: 419-435.

[182] Noort D., Benshop H.P. and Black R.M., 2002. Biomonitoring of exposure to chemical warfare agents: a review. Toxicol. Appl. Pharmacol. **184**:116-126.

[183] Agency for Toxic Substances and Disease Registry (ATSDR), 2003. Toxicological profile for sulphur mustard (update). Public Health Service US Department of Health and Human Service, Washington DC.

SITE	SPECIES	Hg (mg/kg w.w.)
CWAs-impacted site	*Helicolenus dactylopterus* n=20	2.225 ± 0.758
	Conger conger n=20	1.525 ± 0.481
Reference site	*Helicolenus dactylopterus* n=20	0.631 ± 0.136
	Conger conger n=8	0.668 ± 0.481

Table 6–1: Hg concentration (mg/kg wet weight) in *H. dactylopterus* and *C. conger* muscle tissue. Data are expressed as mean ± standard deviation

SITE	SPECIES	As (mg/kg w.w.)
CWAs-impacted site	*Helicolenus dactylopterus* n=20	14.135 ± 8.105
	Conger conger n=20	146.469 ± 46.453
Reference site	*Helicolenus dactylopterus* n=20	3.843 ± 1.916
	Conger conger n=8	39.991 ± 16.422

Table 6–2: As concentration (mg/kg wet weight) in *H. dactylopterus* and *C. conger* muscle tissue. Data are expressed as mean ± standard deviation

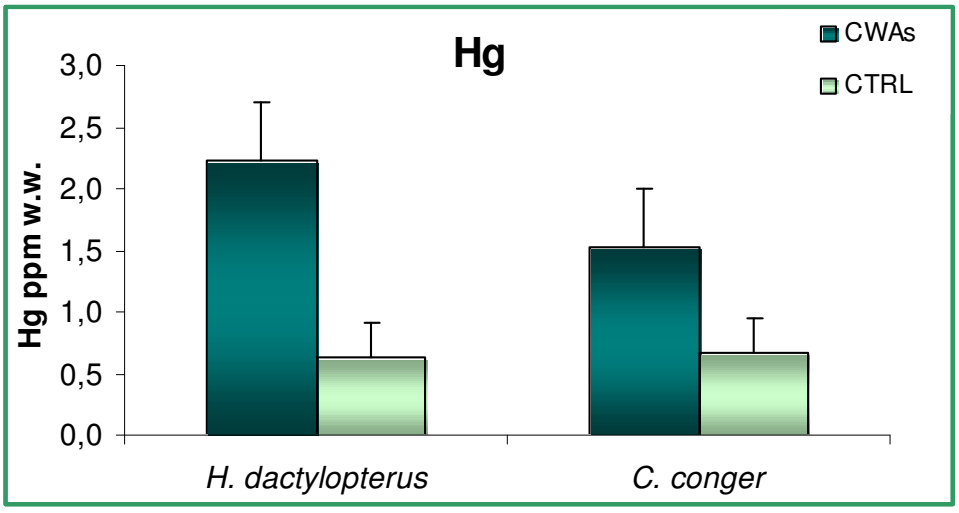


Fig. 6-8 Hg concentration in muscle tissue of *H. dactylopterus* (n=20) and *C. conger* (n=20 in CWAs impacted area; n=8 in reference site)

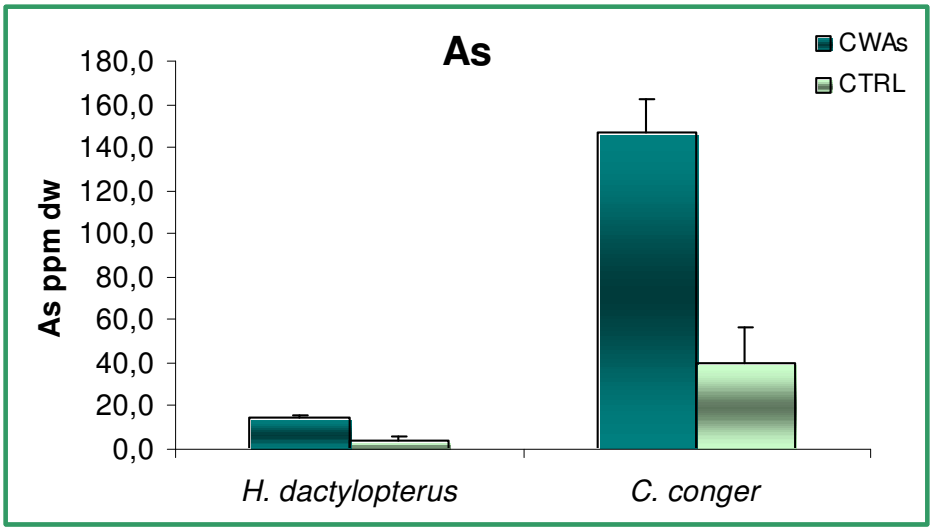


Fig. 6-9 As concentration in muscle tissue of *H. dactylopterus* (n=20) and *C. conger* (n=20 in CWAs impacted area; n=8 in reference site)

6.1.3.3 DISCUSSION

Concerning Hg levels in *H. dactylopterus*, values similar to those measured in the reference site were obtained in individuals from the NW Sardinia and N Sardinia (0.65 and 0.73 mg/kg w.w. respectively)[184] whereas lower levels were detected from Azorean waters (0.29 mg/kg w.w. [185]; 0.26 mg/kg w.w. [186]), according to the anomalous levels of Hg determined in Mediterranean deep-water benthic and demersal fish species[187]. A more recent work reports mean concentration of 0.42 mg/kg w.w. in *H. dactylopterus* captured in the Central and Southern Adriatic Sea[188]. In the light of these findings we can assume that our measurements, determined for the reference site, are in the range of Hg levels for this species in the Mediterranean Sea while concentrations measured in *H. dactylopterus* from the CWAs-impacted site are well above these values.

[184] Renzoni A. and Baldi F., 1975. Osservazioni sulla distribuzione di mercurio nella fauna del Mar Tirreno. Acqua & Aria **8**: 597-6025.

[185] Monteiro L.R., Isidro E.J. and Lopes H.D., 1991. Mercury contents in relation to sex, size, age and growth in two scorpion fish (*Helicolenus dactylopterus* and *Pontinus kulii*) from Azorean waters. Wat. Air Soil Poll. **56**: 359-367.

[186] Andersen J.L. and Depledge M.H., 1997. A survey of total mercury and methylmercury in edible fish and invertebrates from Azorean waters. Mar. Environ. Res. **48** (3): 331-350.

[187] Bacci E., 1989. Mercury in the Mediterranean. Mar. Poll. Bull. **20** (2): 59-63.

[188] Storelli M.M., Giacominelli Suffler R., Storelli A., D'Addabbo R., Palermo C. and Marcotrigiano G.O., 2003. Survey of total mercury and methylmercury in edible fish from the Adriatic Sea. Food Add. Contam. **20** (12): 1114-1119.

Hg tenors reported for the North and the Southern Adriatic Sea are well below the ones measured in *C. conger* collected in the reference and CWAs-impacted sites: 0.129 mg/kg w.w.[189]; 0.3 mg/kg w.w.[190], respectively.

Both *H. dactylopterus* and *C. conger* have shown an important As bioaccumulation in the CWAs-impacted site. Furthermore in *C. conger* As values are definitely higher than values reported for the same species in the Mediterranean Sea (10.33÷17.67 mg/kg w.w. in Ghidini *et al.*, 2000 and 13.86÷145.74 mg/kg dry w. in Sammarini 2004[191]). Storelli *et al.*, 2000[192] report for benthic fish species (e.g. *Raja spp.*), collected in the Southern Adriatic Sea, similar levels to those measured in *C. conger* from the reference site whereas these were clearly below the levels obtained from the CWAs-impacted site.

Comparing the results of As concentration in muscle of *H. dactylopterus* with those obtained within the ACAB project (see par. 5.7.1), the present values result higher, considering also that in the RED COD project the values are expressed in wet weight (Tab. 6–3).

SITE	Project	As (mg/kg)
CWAs-impacted site	RED COD	14.135 ± 8.105 (w.w.)
	ACAB	6.432 ± 2.450 (d.w.)
Reference site	RED COD	3.843 ± 1.916 (w.w.)
	ACAB	4.302 ± 2.561 (d.w.)

Table 6-3: As concentration in muscle of *H. dactylopterus* obtained in fish collected in CWAs impacted area and in the reference area during the ACAB project and the RED COD project

For both species, mercury and arsenic values measured in specimens from the CWAs-impacted site were two to four times higher than those from the reference site and the values measured in benthic fish species collected by other researchers in the Mediterranean sea.

[189] Ghidini S., Delbono G. and Campanili G., 2000. Livelli ed evoluzione di cadmio, mercurio ed arsenico nei pesci dell'Alto Adriatico. Annali Fac. Med. Vet. Univ. Parma.

[190] Istituto Superiore di Sanità, 2004. Rischio chimico associato alla qualità delle acque del mare Adriatico. Rapporto finale delle attività finanziate dal progetto MURST/CNR "Prisma 2", Ferrara F. and Funari E. Eds., Rapporti ISTISAN 04/04.

[191] Sammarini V., 2004. Tenori di contaminanti organici ed inorganici e loro effetti in *Conger conger* e *Paracentrotus lividus* derivanti dalla corrosione di ordigni bellici affondati in mare. Thesis of Degree, Rome University "La Sapienza". Supervisors E. Amato and M. Giovini. 120 pp.

[192] Storelli M.M. and Marcotrigiano G.O., 2000. Organic and inorganic arsenic and lead in fish from the South Adriatic Sea, Italy. Food Add. Contam. **17** (9): 763-768.

Nevertheless our data cannot clearly define the origin of the observed anomalies. Considering the ecological characteristics of the studied species and their possible interactions with compounds leaked from ordnance lying on the seabed we retain possible that the observed high levels of Hg and As could be due to the presence of these elements in the ammunitions. A further enlargement of the study area and chemical-physical characterization of sediments are recommended in order to define the source(s) of the observed high contents of both As and Hg.

6.1.4 Histopathology

The histopathological analyses have been performed on samples collected during the CWs campaigns and the *in vivo* experiment. The analyses were performed to investigate the effects at tissue level eventually determined by yperite or arsenical compounds.

The yperite may cause acute effects resulting in external tissue lesions induced by its vesicant action. The toxicity of sulfur mustard is not only limited to local skin injury: damages of reproductive, respiratory and gastrointestinal systems are reported in several mammalian species, as well as the induction of teratogenesis and cancer. The mechanism of action of sulfur mustard still remains unclear, even though several hypothesis have been reported in literature. At present the most accepted theory ascribes the responsibility for the toxic effects of yperite to the alkylating process of different cell constituents, such as DNA, RNA, proteins and lipid membranes[193]. This reaction could be the cause of physiological, metabolic and genetic failure of cellular functions[194]. The development and the healing of cutaneous injuries induced by yperite have been studied at the microscopic and ultrastructural levels in several animals but no data is available on fish.

Moreover, taking into account that yperite ordnance often contain other toxic chemical compounds, such as arsenic (As), the potential effect of this heavy metal has been investigated as well. As reported in literature, a chronic exposure to arsenic may cause in fish both structural and functional changes in the liver, spleen and gonads[195 196 197 198 199].

Within this study, the effects of acute exposure to sulfur mustard is described for the first time in fish.

[193] Papirmeister B., Gross C.L., Meier H.L., Petrali J.P., Johnson J.B., 1985. Molecular basis for mustard-induced vesication. Fund. Appl. Toxicol. **5**: 134-149.

[194] Kehe K., Szinicz L., 2005. Medical aspects of sulphur mustard poisoning. Toxicology, **214**: 198-209.

[195] Sorensen E.M.B., Mitchell R.R., Harlan C.W., Bell J.S., 1980. Cytological changes in fish liver following chronic, environmental arsenic exposure. Bull. Environ. Contam. Toxicol. **25**: 93.

[196] Sorensen E.M.B. and Smith N.K.R., 1981a. Hemosiderin granules: cytotoxic response to arsenic exposure in channel catfish. Bull. Environ. Contam. Toxicol., **27**: 645.

[197] Sorensen E.M.B., Wenz L.L., Windsor B.C., Mitchell R.R., 1981b. Stereological analysis of arsenic-induced cytological structures. J. Tenn. Acad. Sci., **56**: 131.

[198] Vogelbein W.K., Fournie J.W., Overstreet R.M., 1987. Sequential development and morphology of experimentally induced hepatic melano-macrophage centres in *Rivulus marmoratus.* J. Fish Biol., **31**: 145-153.

[199] Wolke R.E., 1992. Piscine macrophage aggregates: a review. Annual review of fish diseases, **2**: 91-108.

6.1.4.1 MATERIALS AND METHODS

6.1.4.1.1 On field study

Post-mortem exam

During the sampling campaigns the necroscopy was carried out on 67 fishes immediately after capture. The macroscopic analysis has provided:

- biometrical measurements: total length (cm) and weight (g);
- external inspection: skin, gills, fins and eyes;
- internal inspection: observation of viscera *in situ* and examination of each organ;
- tissues sampling for histological analysis.

Health Assessment Index (HAI)

The HAI method proposed by Goede and Burton (1990)[200] and modified by Adams *et al*. (1993; 1996)[201] [202] was applied to evaluate the general health status of wild specimens of *C. conger* and *H. dactylopterus*. The HAI was already applied to assess the health of wild fish population and has been demonstrated as a simple and reliable method to evaluate the effects of environmental pollution in fish. HAI is a quantitative index that allows statistical comparisons of fish health among data sets. Numerical values, based on the degree of severity or damage incurred by an organ or tissue from environmental stress factors, were assigned to index the variables (Tab 6–4).

[200] Goede R.W. and Burton B.A., 1990. Organismic indices and an autopsy-based assessment as indicators of health and condition of fish. Am. Fish Soc. Symp. **8**: 93-108.

[201] Adams S.M., Brown A.M., Goede R.W., 1993. A quantitative health assessment index for rapid evaluation of fish condition in the field. Transaction of the American Fish. Soc.**122**: 63-73.

[202] Adams S.M., Ham K.D., Greeley M.S., LeHew R.F., Hinton, D.E., Saylor C.F., 1996. Downstream gradients in bioindicator responses: point source contaminant effects on fish health. Can.J.Fish. Aquat. Sci. **53**, 2177-2187.

VARIABLE	CONDITION	HI
SKIN	Normal	0
	Mild skin aberrations	10
	Moderate skin aberrations	20
	Severe skin aberrations	30
FINS	No active erosion	0
	Light erosion / haemorrhaging	10
	Moderate erosion / haemorrhaging	20
	Severe erosion / haemorrhaging	30
SPLEEN	Normal: black, dark red, red / granular	10
	Nodules	30
	Enlarged	30
	Other aberrations	30
LIVER	Normal: solid red, light red colour	10
	Fatty liver	30
	Nodules, cysts	30
	Focal discoloration	30
	General discolorations	30
	Other aberrations	30
KIDNEY	Normal: dark red; relatively flat	0
	Enlarged	10
	Mottled / grey discoloration	30
	Granular	30
	Nephrocalcinosis	30
	Other aberrations	30
GILLS	Normal: no apparent aberrations	0
	Frayed; erosion of tips of gill lamellae	30
	Oedema	30
	Discoloured margin	30
	Anaemia	30
	Other aberrations	30
EYES	Normal: good ″clear″ eye	0
	Opacity (one or both)	30
	Swelling (one or both)	30
	Haemorrhaging in the eye (one or both)	30
	Missing (one or both)	30
	Other aberrations	30
PARASITES	Absence	0
	Low presence	10
	Mild presence	20
	High presence	30

Table 6-4: Health Assessment Index parameters and numerical values assigned (modified from Adams et al., 1993)

The numerical values are summed to calculate the HAI value for each fish. The HAI for a sampled population is calculated by summing all individual HAI values and dividing by the total number of examined fish [201].

Histolology

Samples of skin, gills, liver, spleen, kidney and gonads were collected from fish, fixed in Bouin solution (picric acid, formalin and acetic acid) and stored in alcohol 80% prior to analysis. Histological analyses were carried out on different organs selected on the basis of macroscopic lesions detected during autopsy. Additional organs, considered in normal conditions, were analysed as control samples. More than 100 tissue samples were included with an automatic processor (mod. Citadel, Shandon) and embedded in paraffin (Erbaplast, Carlo Erba). Sections (3 microns) were obtained for each sample using a rotative microtome and were stained with haematoxylin-eosin. In some cases different histological stains, such as PAS, blue alcian, Mallory trichrome and Perls were used to analyse specific lesions and the structure of the examined organ. The total number of histological analyses carried out for *C. conger* and *H. dactylopterus* is reported in table 6–5.

	Histological samples (n)						
Species	**Skin**	**Liver**	**Spleen**	**Kidney**	**Gonads**	**Gills**	**Total**
C. conger	9	12	10	10	10	4	55
H. dactylopterus	4	10	10	10	10	4	48
							103

Table 6-5: total number of histological analyses carried out in wild fish (*C. conger* and *H. dactylopterus)* collected in the study area and in the control area

6.1.4.1.2 In vivo *study*

Post mortem exam

Each specimen was subjected to an autoptical analysis to evaluate the heath status as described above. Samples of skin, spleen, liver, kidney and gills were fixed in Bouin solution and transferred in alcohol 80% after 24 h. Histopathological analyses were performed on samples of skin, liver and kidney collected from 42 fishes. (Tab. 6–6).

	Histological samples (n)				
Species	**Skin**	**Liver**	**Kidney**	**Gills**	**Total**
A. anguilla	20	9	9	4	42

Table 6-6: total number of histological samples collected from *A. anguilla* exposed to different concentrations of yperite

Liver profile

Quantitative determinations of lactate dehydrogenase (LDH), glutamic-oxalocetic transaminase (GOT), total protein and total bilirubin in plasma samples were performed by means of a dry chemistry analyzer (SPOTCHEM SP 4410, Menarini).

Statistical analysis

Data were processed with a SPSS software. Due to the limited number of plasma samples, two non parametric tests (the Kruscall Wallace test and the Mann Whitney-test) were applied for statistical comparison of data. Significant level was fixed to $p \leq 0.05$.

6.1.4.2 RESULTS

6.1.4.2.1 On field study

Post-mortem examination

Post-mortem examination showed a compromised health status in fish captured in the study area. In particular, a high number of damages and severe lesions occurred in *C. conger*, which seem to be more sensitive than other fish species. The presence of deep ulcers on skin surrounded by small haemorrhages and nodules along the abdomen and lateral line (Fig. 6–10) were detected in 70% of the analysed specimens of *C. conger* (Tab. 6–7). No significant lesions were observed in specimens of *H. dactylopterus*. In tables 6–8 the results of autoptic examination are reported according to the fishing site, fish species, type of lesion and tissue/organ affected.

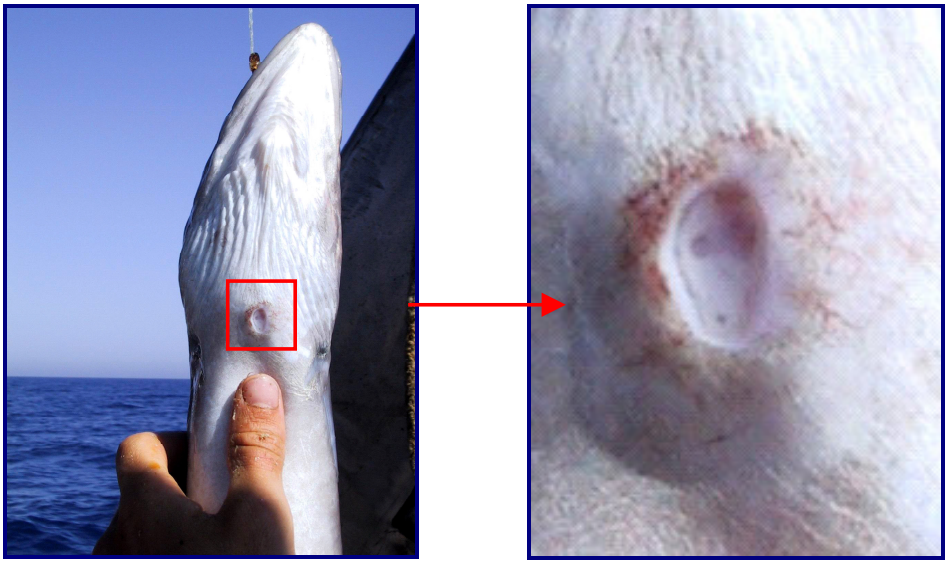
Site **Species** **Organ damaged**	Study area *C. conger* affected fish (%)	**Lesion description**
Skin	70	Lips oedema, reddening and punctiform haemorrhages. Presence of small nodules on abdominal region and beside the lateral line. Some of these were ulcerated. Presence of haemorrhagic and scarred ulcers, haematomas and punctiform haemorrhages around the mandible region. Reddening of anal region
Fins	45	Erosions and haemorrhages
Liver	45	Nodules, change in colour and consistency
Spleen	20	Enlargement and change in consistency

Table 6-7: results of autoptic examination in C. *Conger* collected from the study area

Site **Species** **Organ damaged**	Study area *H. dactylopterus* Affected fish(%)	**Lesion description**
Fins	5	Light erosion with haemorrhages

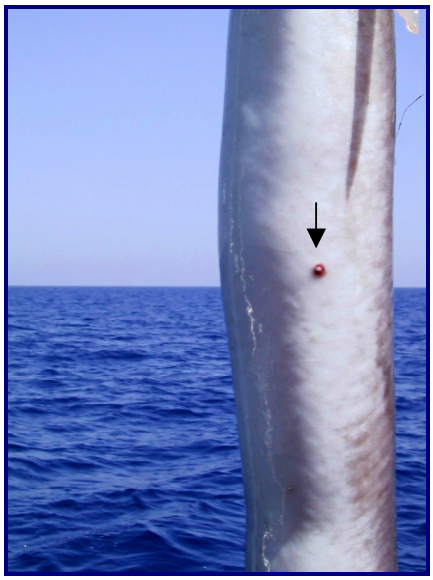
Table 6-8: results of autoptic examination in *H. dactylopterus* collected from the study area

Furthermore both species were parasitized by *Anisakis sp.* (Fig. 6–11). The incidence of *Anisakis* was considerably higher and severe in *C. conger* (65%) than in *H. dactylopterus* (40%).



Skin ulcer. (Ph. Tommaso Petochi, ICRAM)

Skin ulcer. (Ph. Tommaso Petochi, ICRAM)



Skin nodule. (Ph. Tommaso Petochi, ICRAM)

Fig. 6-10 autoptic examination in specimens of *C. Conger* captured in the study area

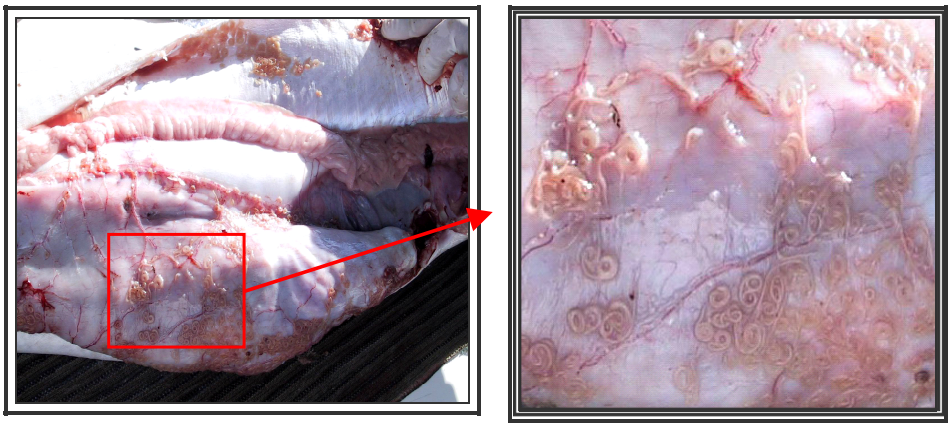


Fig. 6-11: *Anisakis sp.* in body cavity of *C. Conger*: (Ph. Tommaso Petochi, ICRAM)

Fish captured in the reference area did not show any of the clinical signs and /or lesions we suppose sulfur mustard can induce. Light skin lesions caused by the traumatic action of fishing gears were observed in *C. conger*. Their localisation on the lips of fish facilitated the diagnosis. No ulcers and/or nodules as the ones previously described were found. *H. dactylopterus* specimens did not show any significant lesion. The number of fish infested with *Anisakis sp*. as well as the number of parasite per fish was lower in fish captured within the reference area. The list of anatomopathological lesions found in fish captured form the control area is reported in tables 6–9 and 6–10 for *C. conger* and *H. dactylopterus* respectively.

Site **Species** **Organ damaged**	Reference site *C. conger* % in fish	**Lesion description**
Skin	80	Lips oedema with puntiform haemorrhages and reddening. Haematomas and puntiform haemorrhages around the mandible region. Reddening on anal region.
Fins	40	Erosions and haemorrhages
Liver	40	Nodules, change in colour and consistency. Some nodules on the surface were caused by parasites.

Table 6-9: results of autoptic examination in *C. conger* collected from the control site

Site	Reference site	**Lesion description**
Species	*H. dactylopterus*	
Organ damaged	% in fish	
Gills	5	Erosion of tips of gill lamellae

Table 6-10: results of autoptic examination in *H. dactylopterus* collected from the control site

Health Assessment Index (HAI)

The HAI value calculated for fish captured in the contaminated site (Molfetta) were higher compared to HAI values of reference samples (Tab. 6–11), thus indicating that the health status of fish captured in the chemical weapons site was compromised.

STUDY AREA			
C. conger		*H. dactylopterus*	
code	HI	code	HI
C1/04	10	H1/04	30
C2/04	90	H2/04	60
C3/04	70	H3/04	30
C4/05	50	H4/05	30
C5/04	80	H5/04	30
C6/04	60	H6/04	30
C7/04	100	H7/04	70
C8/04	40	H8/04	30
C9/04	110	H9/04	30
C10/04	110	H10/04	60
C11/04	60	H11/04	30
C12/04	90	H12/04	30
C13/04	60	H13/04	30
C14/04	20	H14/04	30
C15/04	60	H15/04	30
C16/04	50	H16/04	30
C17/04	0	H17/04	30
C18/04	70	H18/04	40
C19/04	70	H19/04	30
C20/04	40	H20/04	40
Population	**62.0**	**Population**	**36.0**

CONTROL SITE			
C. conger		*H. dactylopterus*	
code	HI	code	HI
C1/04/S	80	H1/04/S	30
C2/04/S	60	H2/04/S	50
C3/04/S	50	H3/04/S	40
C4/05/S	20	H4/05/S	30
C5/04/S	0	H5/04/S	30
Population	**42.0**	H6/04/S	40
		H7/04/S	30
		H8/04/S	40
		H9/04/S	30
		H10/04/S	40
		H11/04/S	30
		H12/04/S	30
		H13/04/S	40
		H14/04/S	40
		H15/04/S	30
		H16/04/S	40
		H17/04/S	40
		H18/04/S	40
		H19/04/S	30
		H20/04/S	30
		Population	**35.5**

Table 6-11: population HAI of fish collected in the study area and in the control site

Histological analysis

Histological analysis highlighted the presence of multiple lesions in different organs from fish captured within the study area.

Skin: evidence of epidermal necrosis and ulcerations in fish collected in the study area. Ulcers were deep and characterized by proliferation of scarred tissue. In numerous specimens of *C. conger* skin nodules ulcerated on the surface were detected macroscopically. Histological examination pointed out focal epidermal necrosis associated with detachment and loss of tissue (Figures from 6–12 to 6–14). Damage to the upper dermis reflect a typical inflammatory response characterized by vascular endothelial swelling, dermal oedema and inflammatory cell infiltration (Fig. 6–15). In the same subjects similar lesions were healed.

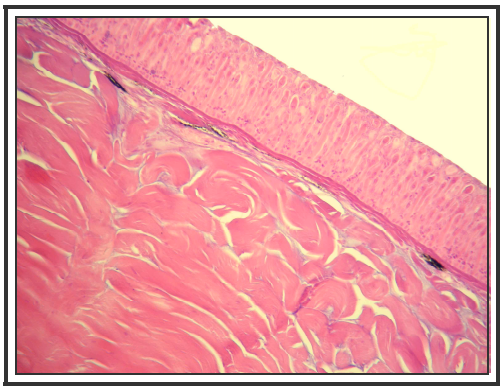


Fig. 6-12 *C. conger* : control skin haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

Fig. 6-13 *C. conger:* skin-process of healing (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

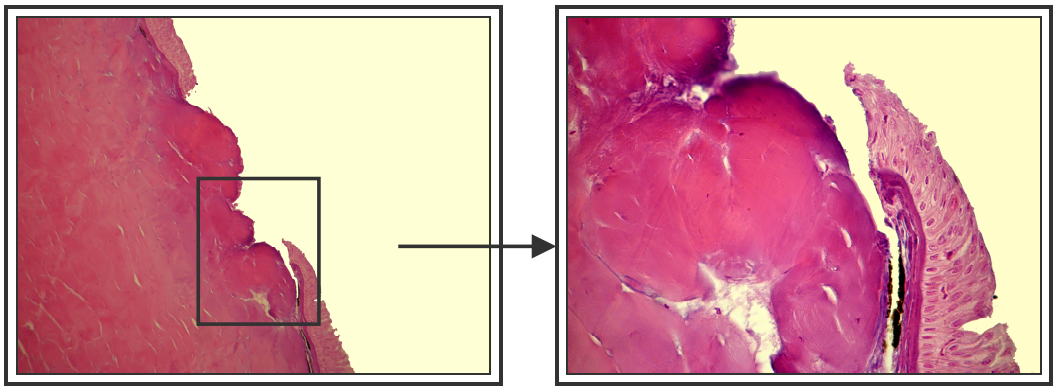


Fig. 6-14 C.conger: skin ulcer (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

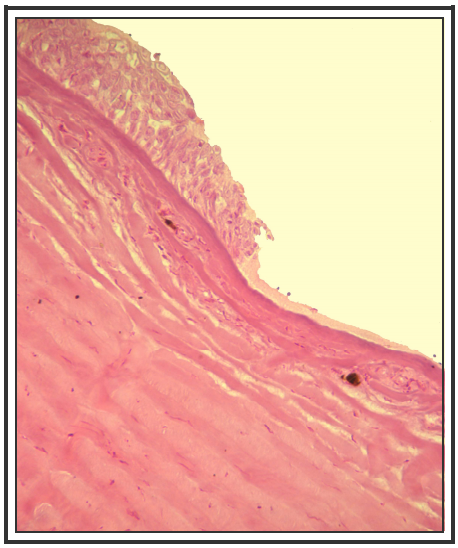


Fig. 6-15 *C. conger*: skin-process of healing (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

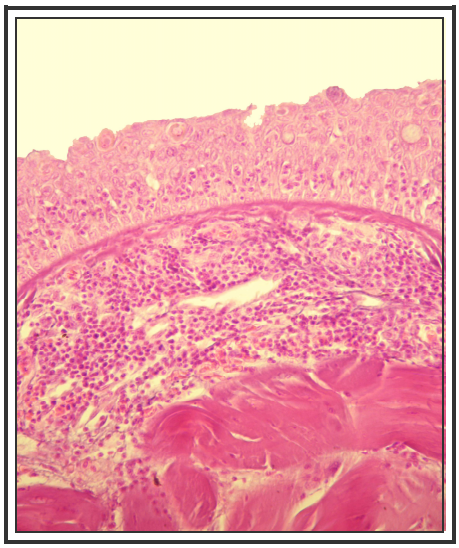


Fig. 6-16 *C.conger*: skin inflammation (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

Liver: tissue samples analyzed from *C. conger* collected in the study area showed the signs of a chronic exposure and lesions of different severeness: serious hyperplasy of bilary ducts (Fig. 6–17), pericholangitis, steatosis and granulomas were observed. A severe periportal fibrosis with changes of centre-lobular hepatocites was pointed out in some specimens (Fig. 6–18). Liver samples from specimens of *H. dactylopterus* captured both in the study and reference site were steatosic. Most fish display a severe degree of steatosis, characterized by a high number of hepatocites with vacuoli in the cytoplasm and the *nuclei* localised in outlying position (Fig. 6–19).

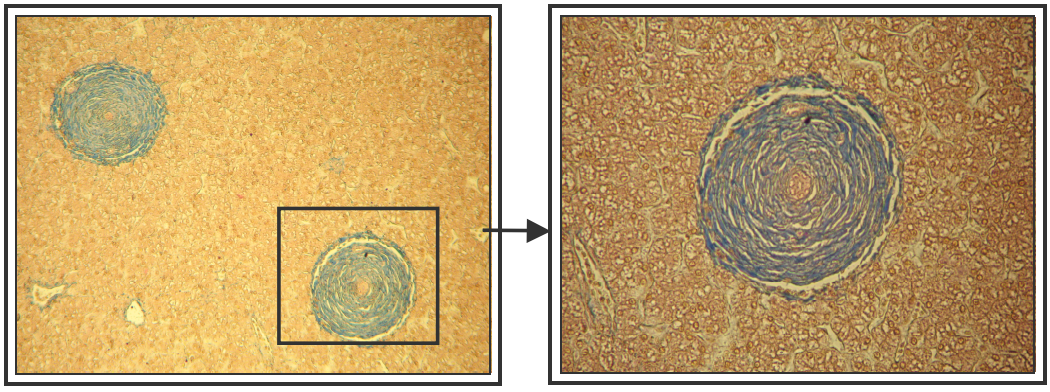


Fig. 6-17 *C. conger*: liver- hyperplasy of bilary ducts (Mallory trichrome) (Ph. Tommaso Petochi, ICRAM)

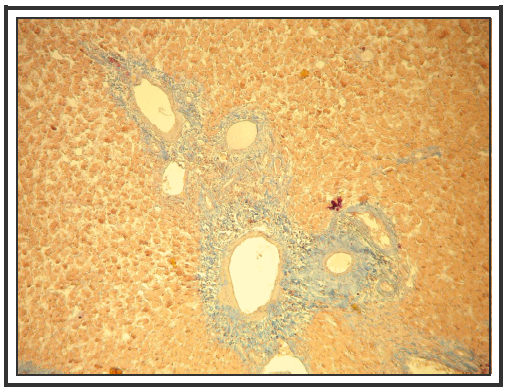


Fig. 6-18 *C. conger*: liver- periportal fibrosis (Mallory trichrome) (Ph. Tommaso Petochi, ICRAM)

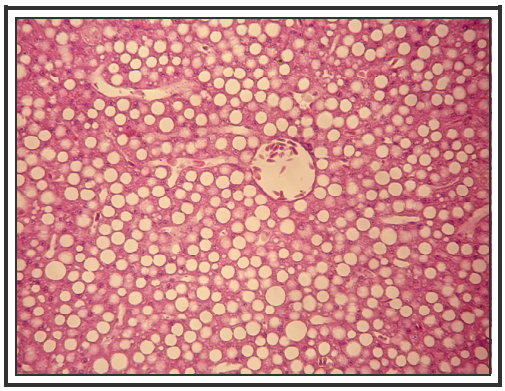


Fig. 6-19 *H. dactylopterus*: liver steatosis (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

Spleen: tissue samples of *C. Conger* from the study area resulted increased in volume. An evident congestion of the red pulp was observed at histological level. Other fish did not display signs of lesions and the distinction between white and red pulp was evident. No difference was found in the number and volume of melano-macrophages centres (MMC) in spleens of both species caught in the study and control sites. Both species did not show any lesion affecting gonads and kidneys. Small haemorrhages were observed on secondary gill *lamellae* as the result of a rapid change of pressure in gill blood-vessels during capture.

6.1.4.2.2 In vivo *study*

Behavioural changes

Some fish exposed to the highest sulfur mustard concentration (10 mg/ml) showed irregular swimming after yperite exposure. The same specimens displayed an anomalous behaviour when trying to bite their flank on the site of injection. This could be ascribed to the high inflammatory reaction on that area. No behavioural change was observed in the other experimental groups.

Post-mortem examination

Results of the *post-mortem* examination showed a slight skin hyper pigmentation around the site of injection in fish injected with the highest yperite concentration (10 mg/ml) after 24 hours exposure. The skin lesions were more evident 48 hours after exposure in fish injected with the high yperite concentrations (1 and 10 mg/ml). In the same group of specimens, also the muscular tissue was degenerated and edematous. After 48 hours, some

specimens of *A. anguilla* exposed to the highest concentration displayed slight skin erosion and haemorrhages in the site of yperite injection. Control groups (Oil, CTRL) and fish exposed to lower yperite concentrations (0.1 and 0.01 mg/ml) did not show any macroscopic lesion throughout the trial.

Fig. 6-20 *A. anguilla*: skin lesion in a specimen sacrified 48 hours after injection with the highest yperite concentration. (Ph. Tommaso Petochi, ICRAM)

Health Assessment Index (HAI)

Population health index of fish exposed to yperite and of control groups are reported in table 6–12. The increase of HAI in the higher yperite concentration (1 and 10 mg/ml) groups is due to the presence of skin lesions described above.

	Population Health Index					
	Control fish		**Exposed fish (mg yperite/ml)**			
	CTRL	Oil	Y4	Y3	Y2	Y1
	Blank	Corn oil	(10 mg/ml)	(1.0 mg/ml)	(0.1 mg/ml)	(0.01 mg/ml)
HI	8.3	8.9	23.0	14.0	8.9	7.8

Table 6-12: population health index of *A. anguilla* specimens exposed to different yperite concentrations

Liver profile

In order to evaluate the potential effects of sulfur mustard and its metabolites on liver functionality different serum parameters, such as total bilirubin, total protein and specific enzymes (glutamic oxaloacetic transaminase - GOT and lactate dehydrogenase - LDH) were measured in plasma samples. Considering the low number of plasma samples available at each sampling time, data from each group were analyzed independently from time of exposure and data from exposed fish were compared with data obtained for the control groups. Total protein content was significantly reduced in fish exposed to the highest yperite concentrations compared to the other fish groups. GOT and LDH levels showed an increase in Y4 and Y3 groups; in the same fishes a slight but not significant increase of plasma bilirubin was also observed.

Parameters	EXPERIMENTAL CONDITIONS					
	CTRL (8)	Oil (5)	Y4 (7)	Y3 (8)	Y2 (9)	Y1 (8)
T-Bil (umol/l)	8.7±0.7	9.6±1.7	12.1±1.9	16±3.5	9.3±0.9	8.6±0.9
T-Pro (g/dl)	4.1±0.2	4.6±0.3	3.5±0.4*	5±0.2	4.6±0.2	4.7±0.2
GOT (IU/l)	179.0±35.2	160.0±22.4	328.0±69.4	314.5±102.9	211.0±36.6	162.5±46.9
LDH (IU/l)	3013.5±468.8	2611.6±644.1	3096.9±470.9	3176.6±364.3	2977.9±455.7	2084.2±486.7

Number of plasma samples are given in parenthesis. Values are expressed by mean ± se.

* Statistically different ($p<0.05$) from other groups.

Table 6-13: plasma liver profiles in *A. anguilla* specimens exposed to different concentration of yperite.

Histological examination

Histological analyses were performed on fish tissues 24 h and 48 h after exposure to yperite. The analyses showed the presence of different lesions and an acute inflammatory response in skin and muscle. The lesions observed were limited to the area of injection. No alterations were found on other target organs such as liver and kidney.

Skin: numerous alterations were observed at epidermal and dermal level among different cell populations. Lesions were much more evident after 48 hours from yperite injection, represented by cytoplasm vacuolization and swelling of club cells in intermediate layer of epidermis in groups Y1 and Y2 (0.1 and 0.01 mg/ml), necrosis and atrophy of the same cells in groups Y4 and Y3 (1 and 10

mg/ml). The *nuclei* of club cells seemed to be particularly sensitive to sulfur mustard. The basal lamina of epidermis was thickened and degenerated in some sections in Y4 fish. In the same fish was observed epidermal necrosis associated with detachment and loss of tissue (Fig. 6–21), discrinia and hyperplasy of mucous cells and melanocytes (Fig. from 6–22 to 6–24). Dermis of eels exposed to the highest sulfur mustard concentration showed degeneration of *stratum compactum* and *stratum spongiosum*. Lesions at this level were characterized by disruption of cell membrane, necrosis and acute inflammatory response with high infiltration of macrophages and neutrophils (Fig. 6–25). Control groups (Oil and CTRL) did not exhibit any lesion at skin level and the different cell populations maintained the proper structure (Fig. 6–26 and 6–27).

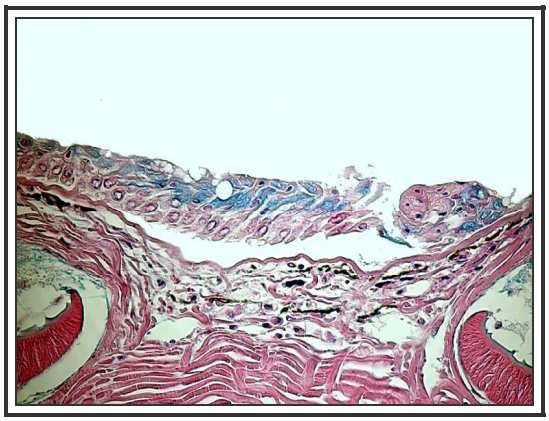


Fig. 6-21 *A. anguilla* (Y4): epidermal necrosis (blue alcian) (Ph. Tommaso Petochi, ICRAM)

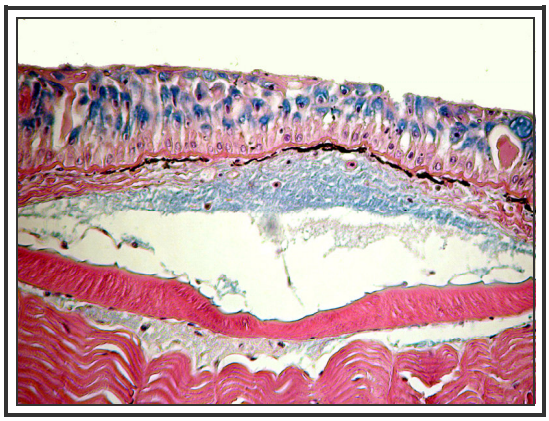


Fig. 6-22 *A. anguilla* (Y4): discrinia and necrosis of club cells (blue alcian) (Ph. Tommaso Petochi, ICRAM)

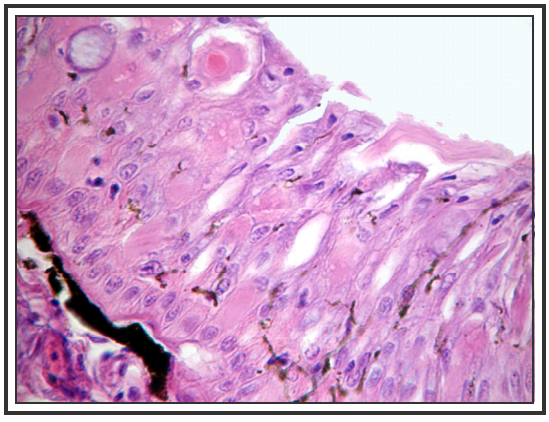


Fig. 6-23 *A. anguilla* (Y3): epidermis-melanocytes iperplasy (haematoxylin-eosin) (Ph. Tommaso Petochi, ICRAM)

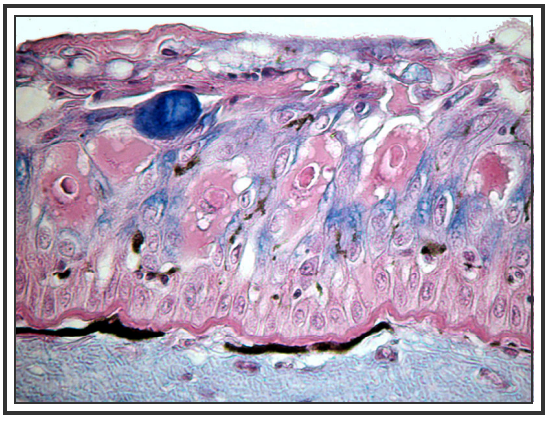


Fig. 6-24 *A. anguilla* (Y1): epidermis- vacuolisation of club cells (blue alcian) (Ph. Tommaso Petochi, ICRAM)

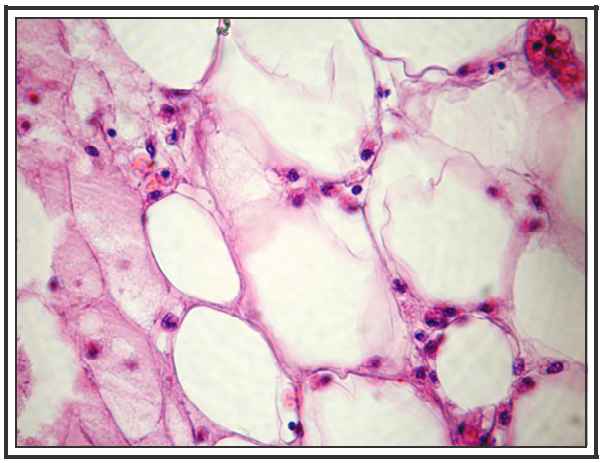


Fig. 6-25 *A. anguilla* (Y4): dermis-inflammation of stratum spongiosum (haematoxylin-eosin). (Ph. Tommaso Petochi, ICRAM)

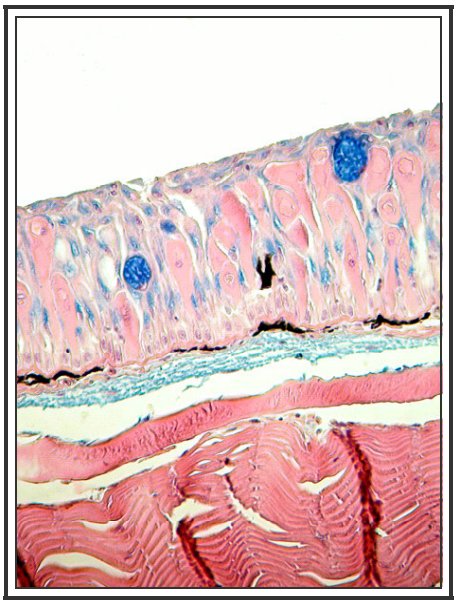


Fig. 6-26 *A. anguilla* (CTRL): control skin (blue alcian)(Ph. Tommaso Petochi, ICRAM)

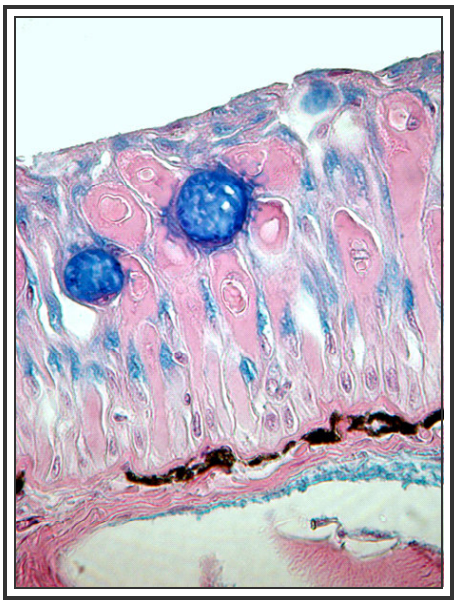


Fig. 6-27 *A. anguilla* (CTRL): control skin (blue alcian)(Ph. Tommaso Petochi, ICRAM)

Muscular tissue*:* samples from Y4 and Y3 groups displayed serious myositis with cellular inflammatory infiltration associated to rhabdomyolisis and colliquative necrosis. Pathological lesions are more severe 48 hours from exposure (Fig. 6–28).

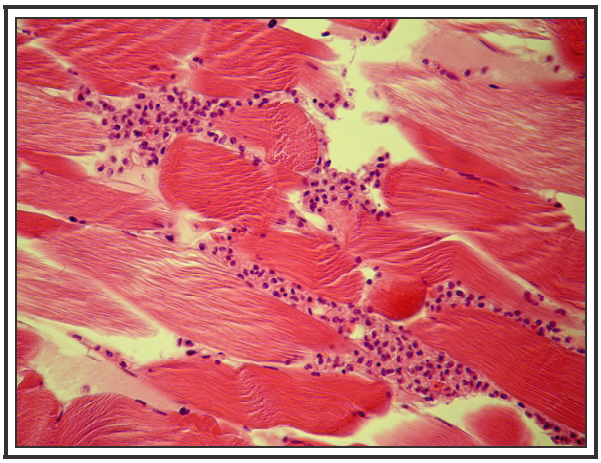


Fig. 6-28 *A. anguilla* (Y4): myositis and necrosis of muscular tissue (haematoxylin-eosin). (Ph. Tommaso Petochi, ICRAM)

6.1.4.3 DISCUSSION

Results of analysis performed on fish captured in the study area, indicate a poor health status of captured target species in comparison with control fish. In particular *C. conger* is the species with the higher HAI, thus indicating a much more compromised health status. All *C. conger* specimens showed numerous lesions in different organs. The skin lesions observed on these fish, could be attributed to different factors. Ulcers discovered in *C. conger* were deep, with regular lips and in phase of healing; therefore the lesions described were neither recent nor caused by the fishing activity. Thus the effect of a toxic compound able to cause skin necrosis and ulcers can not be excluded. The small ulcerated and scared nodules on the skin surface of the same species could be caused by parasites, although no organism has been found at histological level. Furthermore, the possibility that ectoparasites were detached from skin due to the change of pressure during capture can not be ruled out. The typical vesicant action of yperite already reported in numerous animal models[203] [204] [205] [206] [207]

[203] Chauhan R.S., Murthy L.V.R., Arora U., Malhotra P.R., 1996. Structural changes induced by sulphur mustard in rabbit skin. Journal of Applied Toxicology, **16**(6): 491-495.

[204] Chauhan R.S., Murthy L.V.R., 1997. Effect of topically applied sulphur mustard on Guinea pig liver. Journal of Applied Toxicology, vol **17**(6): 415-419.

[205] Brown R.F.R., Rice P., 1997. Histopathological changes in Yucatan minipig skin following challenge with sulphur mustard. A sequential study of the first 24 hours following challenge. Int. J. Exp. Path., **78**: 9-20.

[206] Reid F.M., Graham J., Niemuth N.A., Singer A.W., Janny S.J., Johnson J.B., 2000. Sulfur Mustard-induced Skin Burns in Weanling Swine Evaluated Clinically and Histopathologically. Journal of Applied Toxicology, **20**: 153-160.

[207] Cowan F.M., Yourick J.J., Hurst C.G., Broomfield C.A., Smith W.J., 1993. Sulfur mustard-increased proteolysis following in vitro and in vivo exposures. Cell Biology and Toxicology **9** (3): 269-277.

could be difficult to detect in fish. In fact, as fish do not have the keratin layer over the epidermis [208] the production of vesicles could be prevented.

Livers of *C. conger* collected within the study area display clinical signs of chronic suffering such as fibrosis, hyperplasia of bilary ducts, pericholangitis, steatosis and granulomas. The causes of these lesions may be different, however signs of fibrosis and steatosis are reported in cases of arsenic (As) exposure[209] [210]. Considering that chemical weapons could contain arsenic compounds and taking into account the high level of this heavy metal found in captured fish, a chronic effect on internal organs resulting in a cell damage due to a long-time exposure to this toxic compound can not be excluded.

All specimens of *H. dactylopterus* captured both in study and control site showed hepatic steatosis. In the blackbelly rosefish *Helicoloenus dactylopterus lahillei* the liver is highly involved in the synthesis of a high amount of fatty acids and lipids. In fact the capacity of buoyancy of this fish, which does not have the swim bladder, strongly depends on the quantity of body lipid deposit[211]. This could justify the high amount of lipids found in the liver of *H. dactylopterus* and the diffuse steatosis observed in most fish, independently from the study area.

In other fish species, liver steatosis can be induced by several factors (e.g. diet, toxicant), including the chronic exposure to heavy metals, especially arsenic compounds, as reported for other animals [203].

Sulfur mustard affects the reproductive system in humans and a long term exposure may cause infertility due to the atrophy of testis germinal epithelium and defective of spermatogenesis[212]. However the gonads of both fish species analyzed in this study do not show any sign of lesion reflecting the normal reproductive condition of fish at that season [213] [214] [215].

[208] Henrikson R.C. and Matoltsy A.G., 1968. The fine structure of teleost epidermis. J. Ultrastruct. Res. **21**: 194-232.

[209] Sorensen E.M.B., Mitchell R.R., Harlan C.W., Bell J.S., 1980. Cytological changes in fish liver following chronic, environmental arsenic exposure. Bull. Environ. Contam. Toxicol. **25**: 93.

[210] Sorensen E.M.B., Wenz L.L., Windsor B.C., Mitchell R.R., 1981b. Stereological analysis of arsenic-induced cytological structures. J. Tenn. Acad. Sci., **56**: 131.

[211] Mendez E., Jachmanian I., Grompone M.A., 1993. Lipid distribution in Blackbelly Rosefish (*Helicolenus dactilopterus lahillei*) in relation to its possibile functions as hydrostatic agent and energy reserve. Comp. Biochem. Physiol., **105B** (1): 193-198.

[212] Safarinejad M.R., 2001. Testicular effect of mustard gas. Urology, **58**: 90-94.

[213] White D.B., Wyanski D.M., Sedberry G.R., 1998. Age, growth and reproductive biology of the blackbelly rosefish from the Carolinas, U.S.A. J. Fish Biol., **53**: 1274-1291

Acute exposure to yperite had visible effects in *A. anguilla* in the first 24 h, but injuries appeared to be most severe on the second day, after 48 h exposure. Serious inflammation process, cellular necrosis and significant effects on liver functionality were detected in specimens exposed to the highest level. Vesications are reported to begin on the second day after yperite exposure and may progress for up to 2 weeks[216]. During the experiment, a darkening colour of skin was already visible after 24 h around the injection site and slight erosions of cutaneous cells were already visible after 48 h. Skin of fishes exposed to sulfur mustard at high doses (1 and 10 mg/ml) showed epidermal necrosis and ulceration associated with dermal necrosis, oedema and acute inflammation characterized by infiltration of polymorphonuclear cells, mainly macrophages and neutrophils. Similar lesions are described by Chauhan *et al*. (1996) [203] in rabbits. The activation of several proteases and proinflammatory cytokines seems to be involved in the induction of injury by sulfur mustard. The absence of histological lesions in internal organs, such as liver and kidney, could be retraced to three principal factors: the short time of exposure, the time of metabolism and excretion of sulfur mustard in fish, and the reduction of the yperite action due to the intra-muscular way of injection. In a recent study[217] histopathological evaluations of tissues, performed after yperite exposure, showed that damage was more in the per cutaneous route in comparison with oral and subcutaneous route, assuming a differential metabolism of sulfur mustard at skin level due to the maximum number of metabolically active and rapidly dividing cells. However, the alteration of some liver biochemical parameters such as the increase of GOT and bilirubin and the significant reduction of total plasma protein in the group exposed to the highest yperite concentration, could represent the first signs of cellular disorder and organ suffering. This result is consistent with the one reported by Chauhan and Murty (1997) [204].which observed in rats a significant rise in the levels of GOT after yperite exposure. Furthermore, sulfur mustard can penetrate epithelial tissues easily due to its lipophilic nature and the main part of this toxic compound is rapidly transported away by circulation, causing systemic intoxication besides of

[214] Munoz M., Casadevall M., Bonet S., Quagio-Grassiotto I., 2000 Sperm storage structures in the ovary of *Helicolenus dactylopterus dactylopterus* (Teleostei: *Scorpaenidae*): an ultrastructural study. Environmental Biology of Fishes, **58** (1): 53-59.

[215] O'Sullivan S., Moriarty C., Fitzgerald R.D., Davenport J., Mulcahy M.F., 2003. Age, growth and reproductive status of the European conger eel, *Conger conger (L.)* in Irish coastal waters. Fisheries Research, **64**: 55-69.

[216] Rice P., 2003. Sulphur mustard injuries of the skin: pathophysiology and management. Toxicological Reviews, vol. **22** (2): 111-118.

[217] Vijayaraghavan R., Kulkarni A., Pant S.C., Kumar P., Rao L., Gupta N., Gautam A., Ganesan K., 2005. Differential toxicity of sulfur mustard administered through percutaneous, subcutaneous and oral routes. Toxicology and Applied Pharmacology, **202**: 180-188.

the local damaging capacity[218]. Thus the liver, being the major site of biotransformation and clearance, is an important target of sulfur mustard toxicity. The increase of LDH levels in Y4 and Y3 groups could be explained by the serious necrosis at muscular level after yperite injection.

The first observations made during A.C.A.B. project[219] are confirmed by the present results. However, further analysis on the target species, especially demersal fish species which live in direct contact with sea bottom, should be carried out in other potential contaminated areas. Further analysis of fish health status must be included such as the analysis of the immune status of fish in contaminated site. One of the most dangerous effects of sulfur mustard exposure in mammalians has been determined to be a severe suppression of the immune system which can lead, in case of lesions and blisters, to opportunistic infections, septicaemia and death[220]. Thus the analysis on the immune status of fish could represent a useful indicator able to evaluate their capacity in responding to stress and toxicant agents. In order to reproduce real field conditions, an experimental chronic exposure to yperite should be performed to evaluate the effects of a long-time exposure. Finally, considering that a suitable biomarker for monitoring subacute and chronic exposure to sulfur mustard is still unknown[221] [222], further analysis at ultrastructural level in target fish species could be useful to point out any potential correlation between chronic exposure to toxic compounds and/or environmental stressors.

6.1.5 DNA Damage Tests: Comet Assay and *Micronuclei* test

Since previous studies relieve the potential genotoxic action of yperite, DNA damage tests have been carried out on fish tissues. The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays (e.g. chromosomal aberrations, sister chromatid

[218] Kehe K., Szinicz L., 2005. Medical aspects of sulphur mustard poisoning. Toxicology, **214**: 198-209.

[219] Amato E., Alcaro L., Corsi I., Della Torre C., Farchi C., Focardi S., Marino G., Tursi A., 2006. An integrated ecotoxicological approach to assess the effects of pollutants released by unexploded chemical ordnance dumped in the southern Adriatic (Mediterranean Sea). Marine Biology, **149** (1): 17-23.

[220] Hassan Z.M., Ebtekar M., 2002. Immunological consequence of sulfur mustard exposure. Immunology Letters, **83**: 151-152

[221] Noort D., Benshop H.P. and Black R.M., 2002. Biomonitoring of exposure to chemical warfare agents: a review. Toxicol. Appl. Pharmacol. **184**:116-126.

[222] Chatterjee D., Mukherjee S., Smith G.M., Das K.S., 2004. Evidence of hair loss after subacute exposure to 2-chloroethyl ethyl sulphide, a mustard analog, and beneficial effects of n-acetyl cysteine. J. Biochem. Molecular Toxicology **18** (3):150-153

exchange). Two techniques have been chosen: Comet Assay and *Micronuclei* Test (see parr. 6.1.5 and 6.1.6).

The Comet Assay is gaining popularity over other assays as it can be run rather quickly and is able to clearly detect even low levels of DNA damage (0.1 DNA break per 10^9 Da[223]). The "comet technique" allows to observe different ruptures in the DNA structure produced by toxic compounds, with a consequent change in the electrophoretic mobility of DNA molecules. In a normal situation, a nucleus stained by 4',6-diamidino-2-phenylindole (DAPI) appears at the fluorescence microscope as a somewhat round structure. After damage, the fragments of DNA form a tail (broken DNA) around an intact head and this image resembles a comet shape (see figures below).

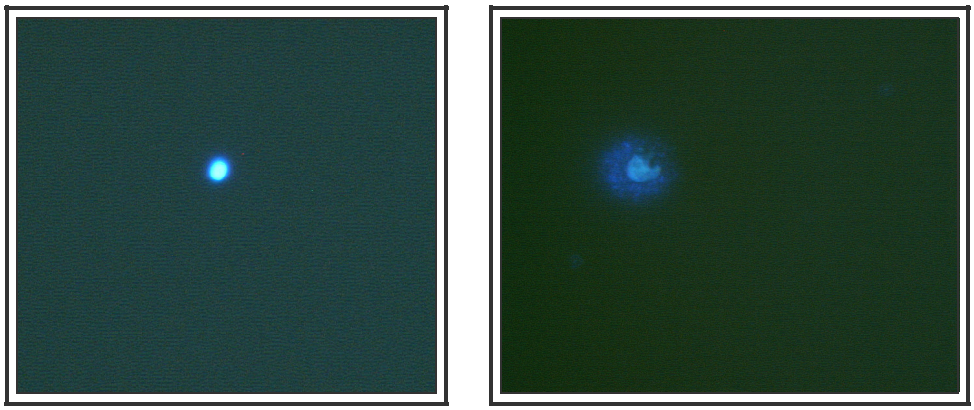


Fig. 6-29 Analysis by Single Cell Gel Electrophoresis (SCGE). Left, a nucleus from gill cell of a specimen of *Conger conger* collected in reference area showing intact structure; right, another nucleus from gill cell of a specimen of *Conger conger* collected in the study area showing a typical comet shape of a damaged DNA. The images were captured by a fluorescence microscope after staining with DAPI (magnification 400X). (Ph. Marilena Di Nardo, CoNISMa)

The Single Cell Gel Electrophoresis (SCGE) comet assay, developed by Singh *et al.*, 1988[224], combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites and crosslinking with the single cell approach typical of cytogenetic assays. The advantages of the SCGE techniques include:

1) the data collection at the level of the individual cell, allowing for more robust statistical analyses;

[223] Gedik C.M., Ewen S.W. and Collins A.R., 1992. Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. Int. J. Radiat. Biol., **62**: 313-320.

[224] Singh N.P., McCoy M.T., Tice R. R., Schneider E. L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res., **175**, 184-91

2) the need for small numbers of cells for sample (<10,000);

3) its sensitivity for detecting DNA damage;

4) virtually any eukaryotic cell population is amenable to analysis.

6.1.5.1 MATERIALS AND METHODS

According to the working protocol, frozen tissues from *Conger conger* and *Helicolenus dactylopterus* (about 50 mg) were washed three times with chilled phosphate-buffered saline (Ca^{2+} Mg^{2+}- free), in order to remove the blood cells, and transferred to ice-cold homogenization buffer (HBSS with 20 mM EDTA, 10% DMSO). The tissues were cut into small pieces using scissors and finally homogenized to obtain a single-cell suspension. The cell suspension was centrifuged at 3000 rpm at 4°C for 5 min and the supernatant was discarded. Viability of the cells was evaluated by the Trypan blue exclusion test method [225]and samples showing >84% cell viability were processed for Comet Assay. About 40 µl of cell suspension (approx. 20,000 cells) was mixed with 75 µl of 0.5% low-melting-point agarose (LMPA) and layered on one end of a frosted plain glass slide, previously coated with a layer of 1% normal agarose (NMA) and a second layer of 0.5% NMA. Finally, it was covered with a fourth layer of 150 µl low-melting-point agarose. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100 added fresh), protected from light and refrigerated at 4°C for a minimum of 2 hours. The slides were then placed in a horizontal gel electrophoresis unit, immersed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA, and 0.2% DMSO, pH 13.5), and left in the solution for 20 min at 4°C for DNA unwinding and conversion of alkali-labile sites to single-strand breaks. Electrophoresis was carried out using the same solution at 4°C for 20 min, using 25 V and 300 mA. The slides were neutralized gently with 0.4 M Tris buffer at pH 7.5.

After the electrophoresis run, the nuclei of the analysed samples were stained with 20 µl of DAPI, and observed by means of a fluorescence microscope equipped with appropriate filters. The images were captured and submitted to analysis using an image analysis system (Scion Image). Thus one square was drawn around the undamaged part of the nucleus (the head of the comet) and one square was drawn around the broken DNA that is visible as a tail. The

[225] Anderson D., Yu T.W-, Phillips B.J., Scheneider P., 1994. The effect of various antioxidants and other modifying agents on oxygen radicals generated DNA damage in human lymphocytes in the comet assay. *Mutat Res,***307**: 261-271.

system gives the measure of the area inside the square in terms of number of pixels.

The parameters selected for the quantification of DNA damage were reported in Tail DNA %, calculated as follows: Tail % DNA = 100 - Head % DNA. About 20 cells for specimen were randomly scored and analyzed.

As for positive control, the lymphocytes were treated *ex vivo* with 100 µM H_2O_2 for 1 h at 4°C.

6.1.5.2 RESULTS

A preliminary analysis of all the available tissues (gill, liver, muscle, kidney, intestine, gonad) of *Conger conger* and *Helicolenus dactylopterus* was performed. These experiments revealed that among the analysed tissues a signal of DNA damage ("comet") was found only in the gills. For this reason the analyses were focused on the breathing apparatus of specimens. A total of 40 samples of gills were analysed by SCGE/Comet Assay: ten samples of *Conger conger* and *Helicolenus dactylopterus* tissues collected within the study area, eight samples of *Conger conger* and ten of *Helicolenus dactylopterus* coming from the reference area.

Results confirmed that all samples of *Conger conger* gills coming from the study area showed comets, indicating the presence of significant DNA damage in this species. Interestingly, a clear "comet" was visible only in the gills of *Conger conger* while no alteration was found in the gills of *Helicolenus dactylopterus*. All other tissues of *Conger conger* analysed by SCGE showed no damage of cells nuclei. None of the control fishes showed significant alteration of the DNA.

The table 6–14 below reports the data for each analysed sample of *Conger conger* collected in the study area.

Specimen Code	Head % DNA	Tail % DNA
M0104C	62.82	37.18
M0304C	79.22	20.78
M0404C	77.85	22.15
M1004C	71.05	28.95
M1104C	68.72	31.28
M1204C	72.41	27.59
M1404C	80.00	20.00
M1504C	65.21	34.79
M1804C	63.54	36.46
M2004C	69.57	30.43
Average + sd	71.49 $\pm$ 6.19	28.51 $\pm$ 6.19
P (t student test)	2.94331e-08	

Table 6-14: quantification of DNA damage in gills of *Conger conger* collected from the study area

As showed in the histogram below there is a dose of Tail DNA % of 28.51% in *Conger conger* gills collected within the study area respect to the controls.

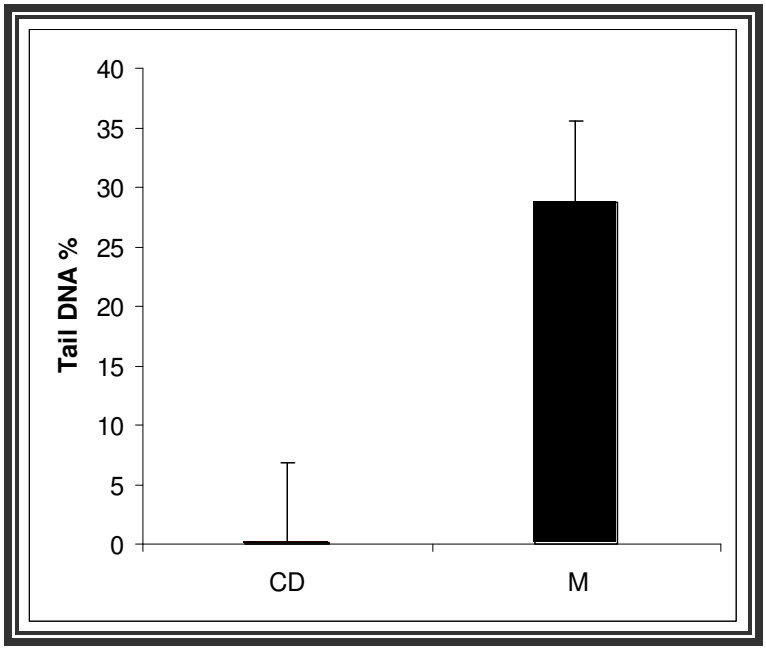


Fig. 6-30 tail DNA% observed in gill tissues of *Conger conger* fished in the study area (M) (n=10) and in the reference site (CD) (n=8)

6.1.5.3 DISCUSSION

The results of the Comet assay show that specimen of *Conger conger* taken from the CWs dumping area displayed a consistent DNA damage in the gills, indicating that chemical agents determine their genotoxic effects especially at the breathing apparatus level. The higher DNA damage in the gill cells could be explained as the gill is the most sensitive target organ that is directly and constantly exposed to the DNA-damaging chemicals dissolved in water[226]. The suitability of gill tissues for genotoxic studies has also been demonstrated using shellfish[227]. Another potential explanation could be that many cells which have undergone an earlier stage of apoptosis, observed in the present study, have contributed to the levels of DNA damage recorded with the Comet Assay[228], while late apoptotic cells are usually not scored with the Comet Assay. This study underlines the effectiveness of the SGCE/comet Assay as a system able to detect genotoxic damages induced by chemical agents as well as its

[226] Dzwonkowska A., Hubner H., 1986. Induction of chromosomal aberrations in the Syriam hamster by insecticides tested in vivo. Arch. Toxicol. **58**: 152-156.

[227] Sasaki Y.F., Nishidate E., Ishibashi S., Tsuda S., Matsusaka N., Asano N., Saotome K., Sofuni T. and Hayashi M., 1997. Detection of genotoxicity of polluted sea water using shellfish and the alkaline single-cell gel electrophoresis assay: a preliminary study. Mutat. Res. **393**: 133-139.

[228] Rank J. and Jensen K., 2003. Comet assay on gill cells and hemocytes from the blue mussel *Mitylus edulis*. Ecotoxicol. Environ. Saf. **54**:323-329.

capability to reveal interspecific differences (*Conger conger* vs *Helicolenus dactylopterus*).

6.1.6 DNA Damage Tests: *Micronuclei* test

Micronuclei test has been considered a very suitable and effective method to use in fish, because of its simplicity and ease of scoring[229] [230].

The *micronucleus* is composed of small chromatin fragments which arise as a result of chromosome breaks after clastogenic action or due to whole chromosomes that do not migrate during the cell division (mitosis) as a result of aneugenic affects[231]. The efficacy of this test system as an indicator of structural genomic damage has already been proven and the *micronucleus* test has been successfully used as a measure of genotoxic stress in fish, under both laboratory and field conditions[232]. This test, based on *micronuclei* counts in actively dividing cell populations, has been utilized in fish as a biological indicator of pollution and for genotoxicity evaluation of physical and chemical agents following direct or indirect exposure *in vivo*. Furthermore, studies indicate that the relative occurrence of *micronuclei* can provide an indication of accumulated genetic damage throughout the life span of the cells even during short phases of contamination. All these considerations suggest the suitability of this test in monitoring the extent of genotoxic damage in marine organisms in a time-integrate manner[233] [234].

The type of mutations that could contribute to micronuclei production include:

- mutations to kinetochore proteins, centromeres and spindle apparatus that could lead to unequal chromosome distribution or whole chromosome loss at anaphase;

[229] Al-Sabti K., Metcalfe C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutation Research, **343**: 121–135.

[230] Çavaş T., Ergene-Gözükara S., 2005. Genotoxicity of metronidazole using the piscine micronucleus test by acridine orange fluorescent staining. Environmental Toxicology and Pharmacology, **19**:107-111.

[231] Heddle J.A., Cimino M.C., Hayashi M., Romagna F., Shelby M.D., Tucker J.D., Vanparys P., MacGregor J.T., 1991. Micronuclei as an index of cytogenic damage: past, present, and future. Environmental Molecular Mutagenesis, **18**:277-291.

[232] UNEP/RAMOGE, 1999. Manual on the biomarkers recommended for the MED POL biomonitoring programme. UNEP, Athens.

[233] Ayllon F., Garcia-Vazquez E., 2001. Micronuclei and other nuclear lesions as genotoxicity indicators in Rainow trout *Oncorhynchus mykiss*. Ecotoxicology and Environmental Safety, **49**:221-225.

[234] Carrasco, K.R., Tilbury K.L., Mayers M.S., 1990. Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. Canadian Journal of Fish Aquatic Science, **47**:2123-2136.

- unrepaired DNA strand-breaks induced by environmental and endogenous genotoxic agents which may results in acentric chromosome fragments.

6.1.6.1 MATERIALS AND METHODS

The aim of this study was to investigate the genotoxic damage, by assessing the frequency of *micronuclei* in blood erythrocytes, in *Conger conger* and *Helicolenus dactylopterus* collected within the study area and in the reference site.

For each animal five peripheral blood smears were immediately made by applying a drop of blood on clean slide, fixed in absolute methanol for 15 min, and air dried. The slides were stained with DNA selective fluorescence dye DAPI (4'-6 diamidine -2 phenyl indole; 1:1000 in 0.1 M citrate buffer pH 7), selectively able to bind DNA. For each specimen, 1000 mature erythrocytes were analyzed under 1000X magnification in order to determine the frequency of *micronuclei*. The analysis was made by an Olympus BX60 microscope equipped with UV apparatus for fluorescence revelation at λ=360 ÷ 400 nm and G365/FT 395/LP420 filter set. Coded and randomized slides were scored using blind review by single observer. The frequency of micronucleated cell was expressed per 1000 cells ($^0/_{00}$). According to Al-Sabti's procedures (1991)[235], we considered as *micronuclei* small fluorescent white-light blue spots, close to the cell nucleus and about one-fifth the size of it (Fig. 6–31 and 6–32).

[235] Al-Sabti K., 1991. Handbook of genotoxic effects and fish chromosomes. Stefan Institute, Ljublijana, Yugoslavia, 221pp.

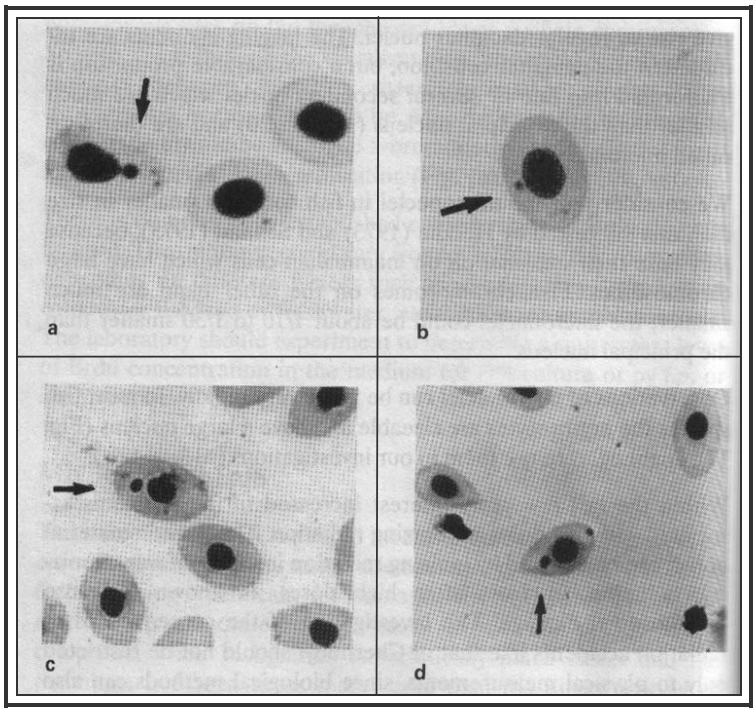


Fig. 6-31 example of *micronuclei* in fish eritrocytes (from Al-Sabti, K.,1991)

In addition, in order to better discriminate *micronuclei*, the following criteria have been strictly applied: 1) only circular or oval spots that were in the same focus of the closest cell nucleus have been taken into account; 2) the spots had the chromatin structure and fluorescence intensity similar to the nuclear one; 3) unhealthy cell nuclei with visible alterations and abnormalities have not been considered.

With reference to the statistical analysis, non-parametric tests were used in order to detect statistically significant differences in the frequency of micronucleated red blood cells between specimens collected in the study and control areas. Kruskal-Wallis test was applied to compare frequencies of *micronuclei* in specimens of *Conger conger* whereas Mann-Whitney test was applied to compare frequencies of *micronuclei* in specimens of *Helicolenus dactylopterus*. In all cases, differences were considered statistically significant when P was $\leq 0,05$.

6.1.6.2 RESULTS

The frequencies of *micronuclei* in red blood cells of specimens deriving from study and control areas are summarised in the table below.

H. dactylopterus				*C. conger*			
MOLFETTA		**CAPO D'ORLANDO**		**CAPO D'ORLANDO**		**MOLFETTA**	
Specimen code	MN	*Specimen code*	MN	*Specimen code*	MN	*Specimen code*	MN
M0104H	0	CD0104H	0	*CD0204C*	1	M1904C	0
M0204H	1	*CD0204H*	1	*CD0304C*	0	*M1304C*	0
M0304H	1	*CD0504H*	0	*CD0404C*	1	*M1804C*	0
M0404H	2	*CD0604H*	1	*CD0504C*	1	*M0104C*	2
M0504H	2	*CD0704H*	2	*CD0604C*	1	*M1504C*	2
M0604H	0	*CD0804H*	2	*CD0804C*	2	*M0704C*	1
M0704H	0	*CD0904H*	1			*T0204C*	1
M0804H	1	*CD1004H*	3			*M0404C*	2
M0904H	1	*CD1104H*	1			*M0504C*	0
M1004H	0	*CD1204H*	0			*M1004C*	1
M1104H	1	*CD1304H*	1			*M1604C*	1
M1204H	6	*CD1404H*	2			*M0904C*	3
M1304H	3	*CD1504H*	0			*M0304C*	1
M1404H	1	*CD1704H*	0			*M0604C*	0
M1504H	1	*CD1804H*	0			*M2004C*	1
M1604H	6	*CD1904H*	1			*M1104C*	0
M1704H	1	*CD2004H*	3			*M1404C*	0
M1804H	1					*M0804C*	1
M1904H	0					*M1704C*	1
M2004H	0						

Table 6-15: frequency of micronucleated erythrocytes (MN expressed per 1000 cells) in specimens of *H. dactylopterus* and *C. conger* from study and control areas

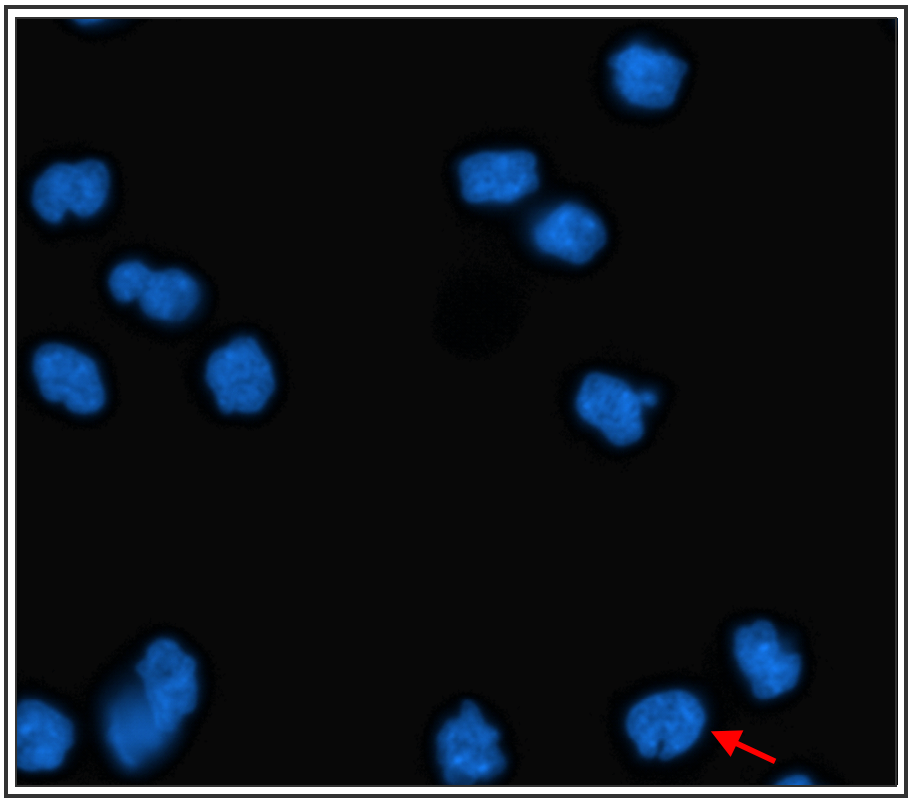


Fig. 6-32 DAPI stained peripheral blood erythrocytes of *H. dactilopterus*.Arrow: *micronucleus* (1000X). (Ph. Milena Modena, ICRAM)

The statistical analysis of *micronuclei* frequencies in samples of *Conger conger* collected in both study and control areas does not show significant differences (H=1.7; g.l. 2; p>0.3; α =0.05; n.s.). The same result was obtained for the statistical analysis of *Helicolenus dactylopterus* specimens (U=164; n 20.17; α =0.05; n.s.).

In conclusion, data indicate that in specimens sampled within the study area there is no evidence of genetic damage at the biological level examined.

6.1.7 Cytochrome P450 system (Stress Index)

In order to observe the eventual damages at a bio molecular level, a study on enzyme activities alteration has been carried out, with particular care for cytochrome. The enzymatic activity may be altered by the presence of xenobiotic substances in the organism. The cytochrome P450 system is the most important multienzymatic complex involved in the first phase of the xenobiotics detoxification process performed by the organism. In Vertebrates it is located on the membranes of the endoplasmic smooth reticulum, mostly in the liver[236].

Cytochrome P450 system (P450) is involved in the detoxification response of the organism to toxic xenobiotics such as PCBs, PAH and nitro aromatic compounds[237] [238] by phase I and phase II enzymes[239] [240] [241]. The phase I consists in the chemical transformation of the hydrophobic compound to a more-water soluble one[242] while phase II involves a conjugation of the parent compound to an endogenous ligand thus facilitating the excretion[243]. Due to its high susceptibility to a specific class of xenobiotics, the P450 system is considered one of the most sensitive biomarkers of exposure in environmental monitoring[237]. The laboratory analyses have been performed as follows:

1) the activity of 7-ethoxy -O-deethylase (EROD), a CYP1A enzyme;

2) total P450 content;

[236] GoksØyr A., Förlin L., 1992. The cytocrome P-450 system in fish, aquatic toxicology and enviromental monitoring. Aquatic Toxicology **22**: 287-312.

[237] Lehman-McKeeman L.D., Caudill D., Vassallo J.D., Pearce R.E., Madan A., Parkinson A., 1999. Effects of musk xylene and musk ketone on rat hepatic cytochrome P450 enzymes. Toxicol Lett. **111** (1-2):105-15.

[238] Van der Oost R., Beyer J., Vermeulen N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Env., Tox., Pharm. **13**: 57-149.

[239] Kleinow K.M., Melancon M.J., Lech J.J. 1987. Biotransformation and induction: Implications for toxicity, bioaccumulation, and monitoring of environmental xenobiotics in fish. Environ Health Perspect **71**: 105-119.

[240] Stegeman J.J., 1981. Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. In POP Ts'o (ed) Polycyclic hydrocarbons and cancer. New York Academic Press, pp 1-60.

[241] Stegeman J.J. and Kloepper-Sams, 1987. Cytochrome P450 isozymes monooxygenase activity in aquatic animals. Env Health Perspect **71**: 87-95.

[242] Bucheli D.T. and Fent K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystem. Env. Sci. Tec. **25**(3): 201-268.

[243] Lech J.J, Vodicnik, M.J., 1985. Biotransformation. In: Rand G.M., Petrocelli S.R., (Eds) Fundamentals of aquatic toxicology; methods and applications. Hemisphere Publishing Corporation, New York, USA, pp.526-557.

3) UDP-glucuronosyl transferases (UDPGT) activity, a phase II enzyme.

The CYP1A1 enzyme EROD is considered the most responsive enzyme to pollutants exposure in fish species including eels[244] [245]: due to its high sensitivity, a measurable modulation of the activity occurs at very low concentrations and in a dose-dependent manner [238] [244]. Total P450 content is considered an additional biomarker being less sensitive of EROD and thus indicating a more serious damage. UDPGT is the most responsive enzyme of phase II. It is considered a valid biomarker in biomonitoring programmes due to its sensitivity to many pollutants [238] [245], although it is less susceptible than phase I enzymes [238].

6.1.7.1 MATERIALS AND METHODS

Preparation of liver microsomes

Livers were homogenized in a 1:4 (w/v) ratio with sucrose buffer (50 mM K_2HPO_4, 0.75 M Sucrose, 1 mM EDTA, 0.5 mM DTT, 400 µM PMSF, pH 7.5) using a Potter-Elvenjem glass/Teflon homogeniser at 2000 rpm. Microsomes were obtained by differential centrifugation in a Sorvall RC28S Ultracentrifuge. Homogenates were first centrifuged at 9000 x g for 20 minutes to remove nuclei, mitochondria, lysosomes and cell debris while the resulting supernatants (S9 fractions) were transferred and centrifuged at 100000 x g for 1 hour. The resulting microsomal pellets were subsequently transferred and resunspended in a 1:2.6 (w/v) solution with Tris-(base) buffer (10 mM Tris), 20% p/v glycerol, 0.5 mM DTT, 400 µM PMSF, pH 7.5). All the procedures were carried out at 4 °C as described by Corsi *et al.,* 2003[246].

Total P450 content

Total P450 was measured by the method of Rutten *et al*, 1987[247] using a Shimadzu UV-160A visible recording spectrometer with the below mentioned

[244] Pacheco M., Santos M.A., 1998. Induction of liver EROD and erythrocytic nuclear abnormalities by cyclophosphamide and PAHs in *Anguilla anguilla* L.. Ecotox. Env. Saf. **40**: 71-76.

[245] Van der Oost R., GoksØyr A., Celander M., Heida H., Vermeulen N.P.E., 1996. Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*) II. Biomarkers: pollution-induced biochemical responses. Aquat. Tox. **36**: 189-222.

[246] Corsi I., Mariottini M., Sensini C., Lancini L., Focardi S., 2003. Cytochrome P450, acetylcholinesterase and gonadal histology for evaluating contaminant exposure levels in fishes from a highly eutrophic brackish ecosystem: the Orbetello Lagoon, Italy. Mar. Poll. Bull. **46**: 203-212.

[247] Rutten A.A.J.J.L., Falke H.E., Catsburg J.F., Topp R., Blaauboer B.J., van Holsteijn I., Doorn L. and van Leeuwen F.X.R., 1987. Interlaboratory comparison of total cytochrome P-450 and protein determinations in rat liver microsomes. Arch. Tox. **61**: 27-33.

assay conditions. The reaction mixture (final volume 2.5 ml): 2440 µl Na_2HPO_4 0.1 M 20% glycerol pH 7.4 at 20°C , 50 µl distilled water, 10 µl of dithionite 1.14 M and 50 µl of microsomal fraction, was gassed with CO for 30 sec. The expression of the total cytochrome P450 content is based on the difference in absorbance in the range between 450nm and 490nm wavelength. Cytochrome peak occurs at 450 nm. Total P450 contained in the microsomal fraction was expressed as nmol·mg·prot^{-1}.

Catalytic assays

The CYP1A enzyme EROD activity was measured according to the fluorimetric methods of Lubet, (1985)[248] using a Perkin-Elmer LS50B luminescence spectrofluorimeter. EROD assay conditions in the reaction mixture (final volume 2.25 ml) were as follows: pH 7.5, 30°C, in a fluorimeter cuvette containing 50 mM Tris-HCl, 25 mM $MgCl_2$ $6H_2O$, 125 µM NADPH and about 50-100 µl of eel liver microsomal fraction. 7-ethoxyresorufin (10 µl 0.1 mgml-1 in DMSO), 0.1 was used as the substrate. The reaction started by adding NADPH and the progressive increase in fluorescence was recorded for 4 minutes at λEX=522 nm /λEM=586 nm. The amount of produced resorufin was calculated from a pure resorufin standard calibration curve with a detection limit of 0.05. EROD activity was expressed as picomoles of resorufin produced per minute per milligram of total microsomal protein (pmol min^{-1} mg prot^{-1}).

UDPGT activity was performed according to Collier *et al*. (2000)[249]. 50 µl of 20 mM 5'-diphospho-glucuronic acid (UDPGA) was added to a reaction mixture containing 50 µl of liver microsomal fraction and 400 µl of 1 µM 4-methylumbelliferon (4-MU) in 0,1 M Tris HCl containing 5mM $MgCl_2$ and 0.05% BSA (pH 7.4) and incubated for 20 minutes at 37°C. The reaction was stopped adding 0.5 ml of trichloroacetic acid and centrifuged 5 min at 3000 rpm. Supernatant was transferred and 1ml of water saturated chloroform was added and vortexed, and the organic phase was discarded. Chloroform wash was repeated twice and 0.25 ml of the supernatant was transferred to a quartz cuvette and added 2 ml of 2 M glycine (pH 10.3). Fluorescence was measured at λ_{EX} = 355 nm /λ_{EM} = 586 nm. Results were expressed as nmol/min^{-1} mg prot^{-1} using a standard curve generated with 4-MU in the range of 900 pM – 4,5 µM.

[248] Lubet R.A., Mayer R.T., Cameron J.W., Nims R.W., Burke M.D., Wolff T., Guengerich F.P. 1985. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. Arch. Biochem. Biophys. **238**: 43-48.

[249] Collier A.C., Tingle M.D., Keelan J.A., Paxton J.W., Mitchell M.D., 2000. A highly sensitive fluorescent microplate method for the determination of UDP-glucoronosyl transferase activity in tissues and placental cell lines. Drug. Met. Disp. **28**: 1184-1186.

Total protein concentrations

Total protein content of the microsomal liver fraction and of the muscle and brain crude homogenate was measured according to Bradford (1976)[250] using a Shimadzu UV-160A visible recording spectrometer and bovine serum albumin as a standard.

Statistical analysis

Data were reported as mean values and standard deviation (SD). Comparison among yperite doses and time of exposure were evaluated by the Mann-Whitney-Wilcoxon rank sum non-parametric test, with significant level set at $p<0.05$. Correlations between parameters were determined with the Pearson correlation coefficient (*r*). Comparison between data of specimens from the CWAs-impacted site and the reference site was evaluated by the ANOVA-MANOVA test. Statistical analyses were performed with Statistica 5.1 (StatSoft, USA).

[250] Bradford M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254

6.1.7.2 RESULTS

In vivo study

The table below reports the main results relating to the laboratory analyses of Total P450 content, UDPGT and EROD activities in the liver of European eels treated for 24 and 48 hours with different concentrations of yperite.

	Time	Corn Oil	0.01 mg/ml	0.1 mg/ml	1 mg/ml	10 mg/ml
TOTAL P450	24h	637.90 ± 84.92	734.21±137.10	566.65 ± 40.92	886.40 ± 442.93	689.65 ± 42.35
nmol/min/mg prot	48h	860.28± 263.36	671.84 ± 88.53	713.94±135.12	909.87 ± 558.14	871.24 ± 344.81
UDPGT	24h	35.95 ± 7.41	28.24 ± 7.42	27.35 ± 5.47	36.08 ± 19.25	35.61 ± 6.24
nmol/min/mg prot	48h	27.16 ± 1.53	36.00 ± 6.42*	36.87 ± 7.64*	38.65 ± 5.13*	33.68 ± 1.92
EROD	24h	148.46 ± 62.03	233.81 ± 3.86*	233.80 ± 43.86	216.84 ± 79.65	307.01 ± 127.43
pmol/min/mg prot	48h	245.26± 50.63	410.41 ± 2.77*	402.30±137.06*	262.53 ± 100.43	309.35 ± 216.45

* Indicates statistical differences from control ($p<0.05$)

Table 6-16: hepatic P450 enzymes in European eel exposed to yperite. Values are expressed as mean ± s.d. n=5

A significant increase in EROD activity was observed in eels exposed to the lowest concentration of mustard gas (0.01 mg/ml) both after 24 h (222%) and 48 h (167%) of exposure; at 0.1 mg/ml EROD activities increased only after 48 h post injection (166%). On the opposite, the highest dose of mustard gas did not produce any effect on EROD activity (Fig 6–33).

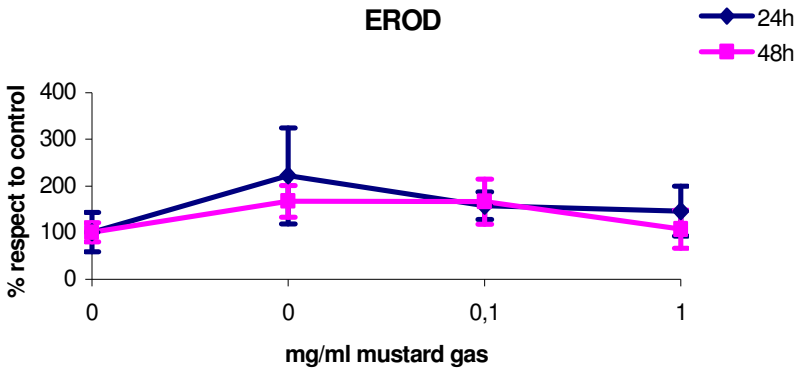


Fig. 6-33 EROD activity (expressed in % respect to control) in liver of European eels treated for 24 or 48 hours with different concentrations of yperite (n=5)

No differences were observed in P450 content in mustard gas treated eels and controls. No modulation of UDPGT activity was evident after 24 h while it increased significantly after 48 h in all treated eels: 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml (142%, 136% and 133% respectively). No further increase of UDPGT activity was observed in eels treated with the highest dose of mustard gas (10 mg/ml) (Fig 6–34).

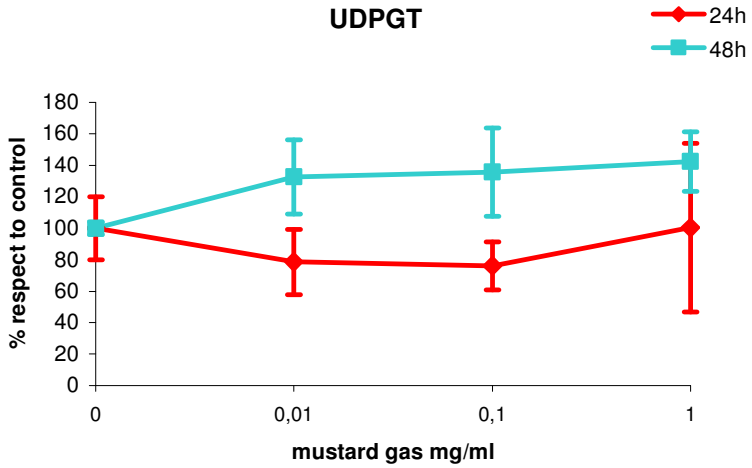


Fig. 6-34 UDPGT activity (expressed in % respect to control) in liver of European eels treated for 24 or 48 hours with different concentrations of yperite (n=5)

On field study

Both *Conger conger* (European conger) and *Helicolenus dactylopterus* (Blackbelly rosefish) from the CWAs-impacted site (Southern Adriatic sea) showed significant higher hepatic EROD activities compared to specimens from the reference site (Southern Tyrrhenian sea) ($p<0.01$). On the opposite, total

P450 content was significantly lower in specimens of the CWAs-impacted site when compared to the reference site.

The main results related to the laboratory analyses of Total P450 content, UDPGT and EROD activities are reported in the following table.

	C.conger		
	Total P450 nmol/min/mg prot	UDPGT nmol/min/mg prot	EROD pmol/min/mg prot
CWAs-impacted site (n=20)	415.33 ± 115.02*	30.26 ± 7.83	401.68 ± 168.58*
Reference site (n=6)	737.37 ± 254.86*	35.07 ± 7.17	50.64 ± 17.41*
	H. dactylopterus		
	Total P450 nmol/min/mg prot	UDPGT nmol/min/mg prot	EROD pmol/min/mg prot
CWAs-impacted site (n=20)	810.39 ± 104.05*	65.53 ± 29.93	26.68 ± 14.07*
Reference site (n=20)	1068.58 ± 188.91*	50.87 ± 14.87	9.68 ± 4.11*

* Indicates statistical differences from control ($p<0.05$)

Table 6-17: total P450 content, UDPGT and EROD activities in liver of *Conger conger* (European conger) and *Helicolenus dactulopterus* (Blackbelly rosefish) collected both in CWAs-impacted site and in reference site.

***Values are expressed as mean ± s.d.**

European conger showed more differences in both EROD activity and total P450 content than blackbelly rosefish. No differences in the UDPGT activities were observed in both species from the impacted and the reference sites (Figures 6–35 and 6–36).

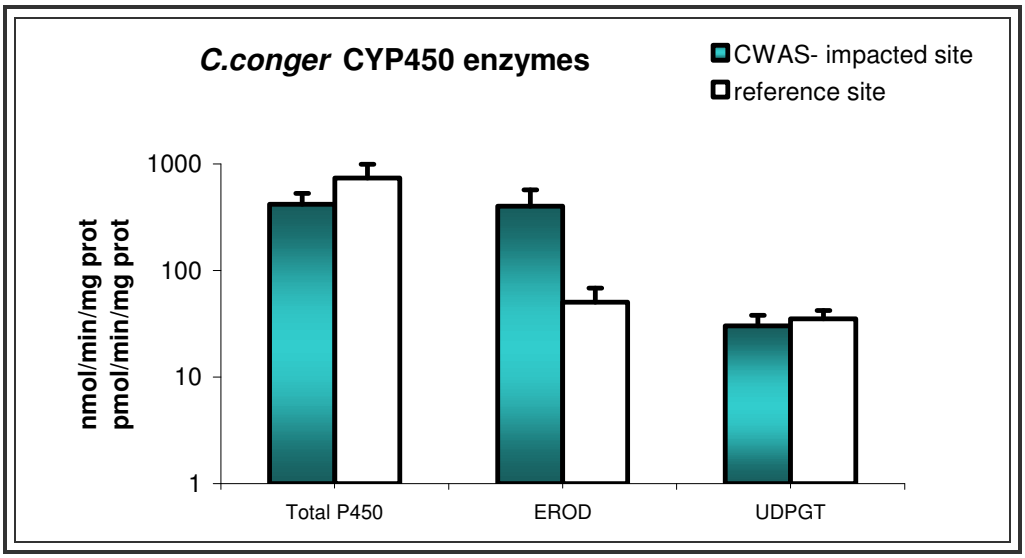


Fig. 6-35 total P450 content, UDPGT and EROD activities in liver of *Conger conger* sampled in CWAs-impacted area (n=20) and in reference site (n=6)

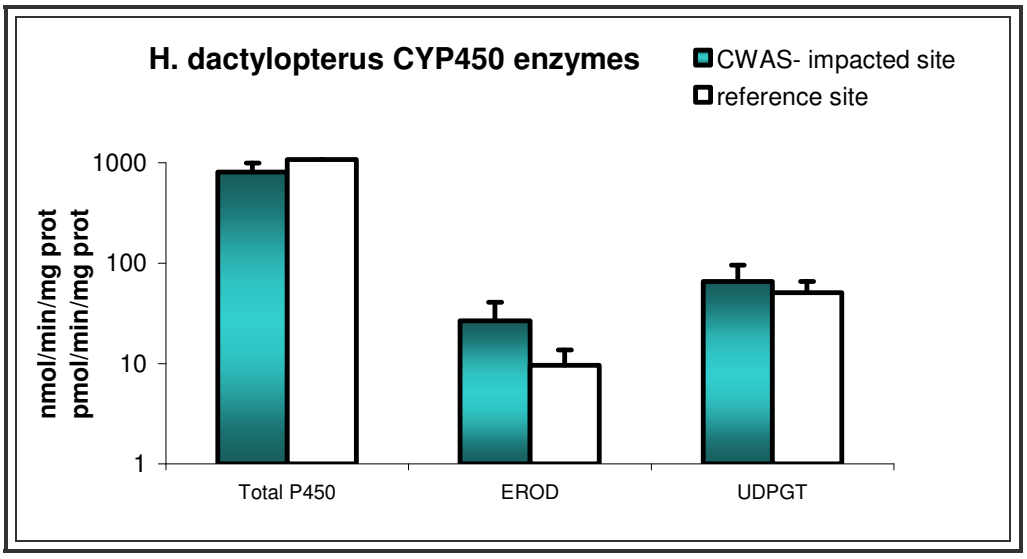


Fig. 6-36 total P450 content, UDPGT and EROD activities in liver of *Helicolenus dactylopterus* sampled in CWAs-impacted area (n=20) and in reference site (n=20)

6.1.7.3 DISCUSSION

While no data are available regarding the effects of mustard gas on fish CYP450, controversial data have been reported in mammals. Pons *et al.*, (2001)[251] reported a significant increase in CYP3A1 expression in lungs of rats despite no induction was observed for both CYP1A and 2E families. An increase

[251] Pons F., Calvet J. H., Haag M., Raeppel V., Keravis T., Frossard N., 2001. Altered expression of lung cytochrome P-450 3A1 in rat after exposure to sulfur mustard. Pharm and Techn., **88** (1).

in P450 content in liver of mice exposed to thiodiglycol and a decrease in P450 content following mustard gas administration were observed while no CYP forms seemed to be involved in thiodiglycol metabolism in humans[252].

The results of our *in vivo* experiment show controversial results as well: a significant modulation of EROD and UDPGT activities due to mustard gas at the lowest concentrations while for total P450 content no alteration was observed. No dose-dependent induction of EROD and UDPGT activities was observed. In particular EROD activity seemed to decrease with increasing doses of mustard gas. Due to the high reactivity of mustard gas and to its high rate of degradation once entered the organism[253], the observed variation of EROD and UDPGT activities could be ascribed also to mustard gas degradation compounds.

Further studies should investigate whether a different administration route for mustard gas such as an intraperitoneal injection or via water may affect differently the cytochrome P450 responses of the organism.

On the other hand a clear result came from the *in situ* study. Both European conger and Blackbelly rosefish from the CWAs-impacted site show significant higher EROD activities compared to specimens from the reference site ($p<0.01$), with differences more evident in the European conger than in the blackbelly rosefish. On the opposite, total P450 content was significantly lower in specimens from the CWAs-impacted site when compared to the reference site. The same for UDPGT activity, although differences were not statistically significant.

From the overall data, despite the different biology and ecology of the two selected sentinel species, the European conger and the Blackbelly rosefish seem to exhibit similar P450 responses, as already reported in a previous study carried out in 1999 on blackbelly rosefish collected in the same area (ACAB project) (see par. 5.7.1). By comparing the results of EROD values in liver of *H. dactylopterus* with those obtained within the ACAB project, the values are higher in the present project.

[252] Hodgson E. , Rose R. L. 2005. Pesticide metabolism and potential for metabolic interactions. J Biochem Mol Toxicol. **19**:276-7.

[253] Noort D., Benshop H.P. and Black R.M., 2002. Biomonitoring of exposure to chemical warfare agents: a review. Toxicol. Appl. Pharmacol. 184:116-126.

SITE	Project	EROD (pmol/min/mg prot)
CWAs-impacted site	RED COD	26.68 ± 14.07
	ACAB	16.454 ± 8.08
Reference site	RED COD	9.68 ± 4.11
	ACAB	8.054 ± 5.87

Table 6-18: EROD activity of *H. dactylopterus* collected in the study area and in the reference site during the ACAB project and the RED COD project

A suitable use of EROD as a valid biomarker of exposure for environmental biomonitoring studies[254] of mustard gas exposure may thus be hypothesised.

6.1.8 Discussion of Laboratories results

The overall results of the integrated ecotoxicological approach highlight several concerns regarding the presence of rusted bombshells and CWAs leakage on the benthic ecosystems of the South-East Adriatic dumping site. The alterations registered at biochemical, cellular and tissue levels within the sampled organisms have been evidenced by a multidisciplinary approach. Moreover, the high As tenors, the observations of dermal blisters as well as the relieved DNA damage allow us to suppose that yperite and arsenical compounds could be the main toxic agents suspected to generate such negative effects.

Although neither CWAs nor their metabolites were detected in fish, in agreement with their reported low persistence in organism tissues (less than 10%), the high status of corrosion of bombshells on the sea floor suggests a continuous input of CWAs into the marine environment thus making the contemporary presence of CWAs and their by-products within the impacted sites reasonable. In this view also their bioavailability might increase.

Moreover, the results obtained clearly indicate that the Conger eel is a very useful bioindicator for CWAs pollution, more than the Blackbelly rosefish. Probably the main reason for this is the particular behaviour of Conger eel which generally lives in holes of the hard sea bottom. Within the study area in fact, characterised by an incoherent substratum, these organisms have been

[254] Amato E., Alcaro L., Focardi S., Marino G., Tursi A., Farchi C., Corsi I., Borghini F., 2001. Fate of persistent chemical warfare agents in a benthic ecosystem of the Southern Adriatic Sea. 36th CIESM Congress Proceedings. Rapp. Comm. int. Mer Médit., 36: 105, Monaco.

observed living inside or underneath the rusted bombs as if the ordnance were effectively part of the seafloor (direct observation through ROV during ACAB survey). As a consequence, there are more possibilities for specimens of *Conger conger* to be affected by the relevant pollutants.

6.2 TNT: laboratory analyses

6.2.1 *In Vivo* Test

Although several studies have been already performed in order to assess the ecological risk due to the presence of TNT and its biodegradation products in the environment, most of the toxicological data used to characterize the effects of these compounds (reduced survival, growth and reproduction) arise from laboratory experiments. Little is known on the biochemical alteration occurring in fish exposed to these compounds.

In order to acquire more information on this topic as well as to verify *in vivo* the results of the *in situ* study, 40 specimens of the European eel *A. anguilla* L. (mean total length 34.64 ± 2.69) were collected using fish traps positioned in few sites throughout the Orbetello lagoon (Tuscany, North Western Mediterranean Sea) and shipped to the laboratory in oxygenated water tanks. Eels were acclimatized three days in 500 l aquarium at 35‰ salinity and 22°C temperature prior to the experiment and then were put into 40 l aquarium. Fishes were divided into 5 groups of 8 specimens each:

- three groups were maintained in aquariums spiked with 0.5, 1 and 2.5 mg/L nominal concentrations of TNT using Dymethylsulfoxide (DMSO) 0.1‰ as a carrier;
- one group was placed in an aquarium spiked only with the carrier;
- one group was kept untreated in order to reveal potential effects due to DMSO or handling conditions.

Fish were sacrificed after 6 and 24 hours through beheading. Tissues were collected to analyse the content of TNT and its degradation products and for stress indexes evaluation. Each specimen was subjected to autoptical analysis, measured and weighted, then muscle, liver, blood and gall bladder were removed and stored in liquid nitrogen or at -20°C for stress indexes evaluation and TNT content. Blood was sampled from the caudal vein by a heparinyzed syringe for chemical analysis and stored in heparinysed tubes.

The main laboratory results obtained are described in detail hereafter.

6.2.2 TNT and its Degradation Products in Fish Tissues

6.2.2.1 MATERIALS AND METHODS

The analysis of TNT and its degradation products within the tissues of the sampled organisms has been carried out following Ek *et al.*[255]. As the bibliographic research revealed the low bioaccumulation rate of TNT in organisms, before starting the analyses of samples collected from the *in vivo* test, five specimen of *Anguilla anguilla* have been contaminated by a dermal injection of TNT in corn oil (solvent) and sacrificed after 6 hours. TNT concentration was 500 ppm respect to the body weight.

The analyses were performed on plasma samples. First 900 units of B-glucuronidase (type H1 from *Helix pomatia*) were added to 25 µl plasma in 1.1 ml tapered screw-top vials, and the samples were incubated at 37°C for 22 hours. After the incubation, 10 µg of hexachlorobenzene, the quantification standard, was added to each sample. In order to extract the analytes, 500 µl of hexane was added and the samples were sonicated for 30 minutes. Then 250 µl of hexane phase of each sample was transferred to 400 µl inserts in 2 ml screw top vials with pasture pipettes.

The chemical analyses were performed on a combined gas chromatograph and mass spectrometer (GC-MS): POLARIS Q Thermo Electron. The GC was equipped with a 30 m × 0,25 mm fused silica column coated with 1-MS (100% polimethyl siloxane, df=0.25 µm). Temperature programming was 30 °C for 3 min; 10 °C/min to 250 °C, followed by isothermal at 250 °C for 10 min. Injector temperature was 225 °C and the transfer line was programmed at 225 °C for 20 min and 10 °C/min to 250 °C, and kept isothermal. Helium was used as carrier gas, at 30 cm/sec., and the electron impact (EI mode) mass spectra were obtained at 70 eV. Limit of detection (LOD) for the analysis of TNT and derivatives was less than 5 pg/µl and the limit of reliable quantification (LOQ) was 10 pg/µl. Compounds were identified by their GC retention times and obtained mass spectra and compared with authentic samples of synthetic references. Mass spectra were also compared to commercially available MS libraries (NIST).

[255] Ek H., Dave G., Birgersson G., Forlin L., 2003. Acute effects of 2,4,6 trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout. Aquatic Ecosystem Health management, 6 (4): 415-421.

2 amino-4,6 dinitrotoluene (2A), 4 amino-2,6 dinitrotoluene (4A) and TNT have been used as standards which allowed to obtain the calibration curve. With this aim four solutions at the concentration of 0.5, 5, 10 and 20 mg/L respectively have been prepared. Mass spectrometry detector response shows a linear correlation between concentration and signal intensity (Fig. 6–37).

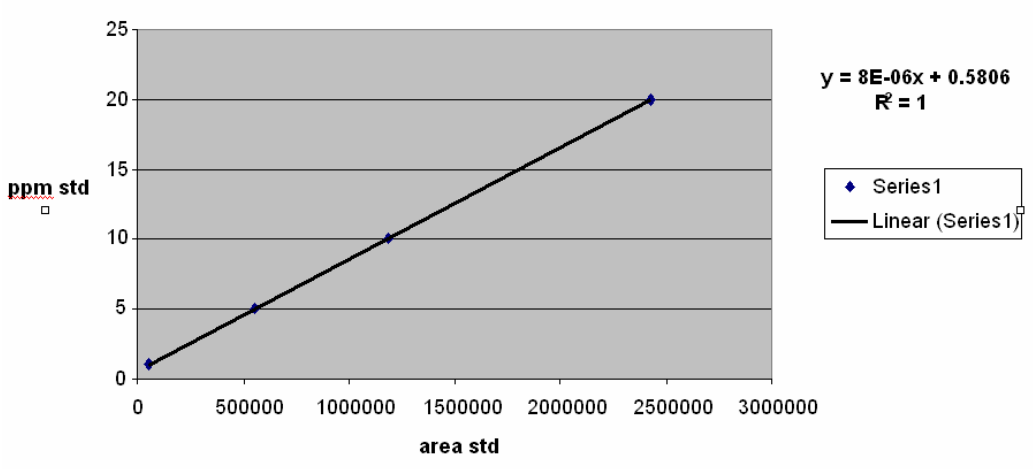


Fig. 6-37 TNT calibration curve in acetonitrile

6.2.2.2 RESULTS

Chromatographic peak retention times have been examined in order to reveal eventually peaks of yperite and its degradation products. There is no evidence of compounds of interest in fish tissue samples (Fig. 6–38).

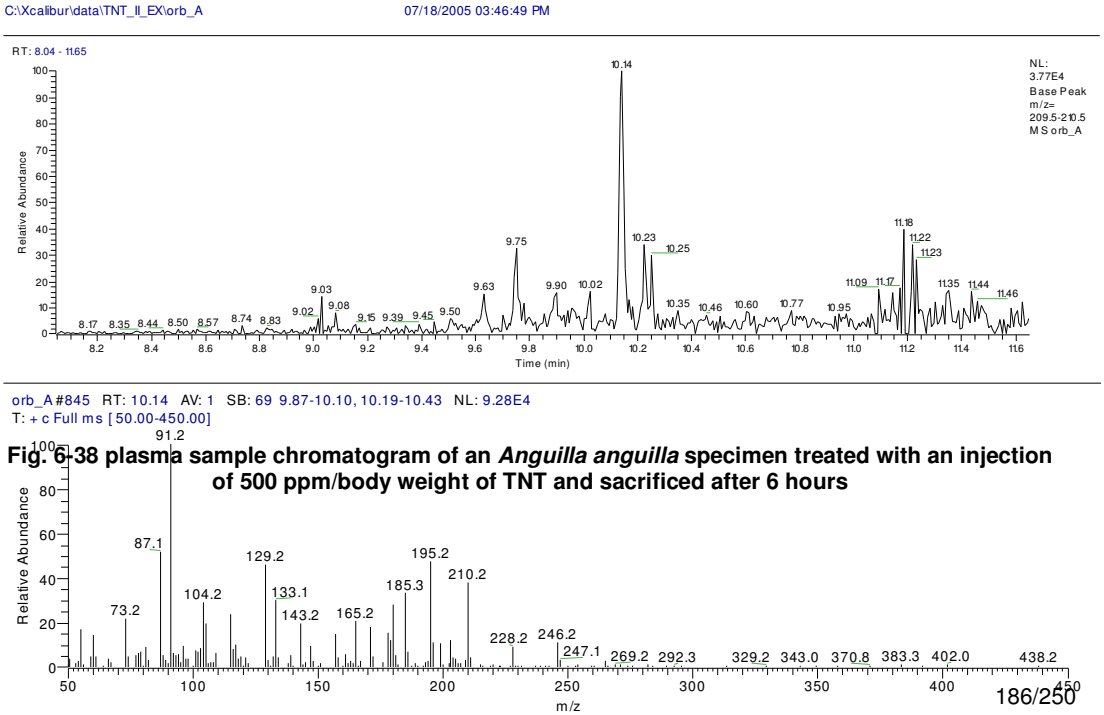


Fig. 6-38 plasma sample chromatogram of an *Anguilla anguilla* specimen treated with an injection of 500 ppm/body weight of TNT and sacrificed after 6 hours

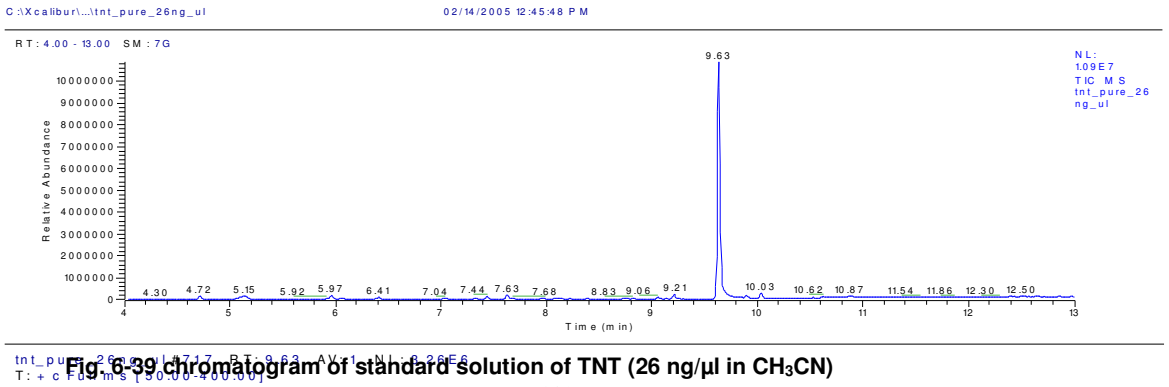


Fig. 6-39 chromatogram of standard solution of TNT (26 ng/µl in CH_3CN)

6.2.2.3 DISCUSSION

The results do not exclude the presence of these molecules in the impacted site. The absence of detectable traces could be explained by the fact that, once released in the marine environment, most of the TNT tends to bind to the organic and clay fraction of sediment and at the same time it is highly metabolized within 1 h of exposure[258]. Consistent with this explanation, other laboratory and field researches, obtained similar results [257] [259].

6.2.3 *Micronuclei* test

The micronuclei test has been carried out following the same procedure as described in the previous chapter regarding CWAs. As for CWAs, data indicate that in specimens sampled within the study area there is no evidence of genetic damage at the biological level examined.

6.2.4 Cytochrome P450 system and ChE (Stress Indexes)

As more detailed in paragraph 6.1.7, related to laboratory analyses of yperite, the analyses on the alteration of enzyme activities have been carried out also for the research on TNT. Particular attention has been given to the cytochrome P450 system in the liver and to the enzyme Cholinesterase (ChE) in the muscle. Previous studies focused on TNT interactions with P450 enzymes in

[256] Green A., Moore D., Farrar D., 1999. Chronic toxicity of 2,4,6-trinitrotoluene to a marine polychaete and an estuarine amphipod. Env. Tox. Chem., **18** (8): 1783-1790.

[257] Lotufo G.R., Farrar D.J., Inouye L.S., Bridges T.S., Ringelberg D.B., 2001. Toxicity of sediment-associated nitroaromatic and cyclonitramine compounds to benthic invertebrates. Environ. Tox. Chem. 20 (8): 1762-1771.

[258] Conder J.M., Lotufo G.R., La Point T.W., Steevens J.A. 2004. Recommendations for the assessment of TNT toxicity testing in sediment. Environ. Toxicol. Chem. 23:141-149.

[259] Scottish Office Agriculture, Environment and Fisheries Department (SOAEFD), 1996. Surveys of the Beaufort's Dyke explosives disposal site, November 1995-July1996. Fisheries Research Services Report No 15/96. 104pp..

Vertebrates[260] [261] [262] [263] have been carried out but no data are currently available on marine fish species. With respect to the analyses aimed at assessing the effects of yperite, within this research the activities of additional enzymes have been taken into account. Other than the EROD activity, the total P450 content and the UDPGT activity, additional laboratory analyses have been carried out as follows:

1) the activity of 7- methoxyresorufin-O-deethylase (MROD), CYP1A enzymes like EROD;

2) the activity of NADPH cytochrome c reductase;

3) 7-benzyloxy-resorufin (BROD) activity, a CYP2B-like enzyme;

4) Cholinesterase (ChE).

ChE is a specific marker for neurotoxic effects being an enzyme responsible for the degradation of the transmitter acetylcholine (Ach) in the cholinergic synapses and neuromuscular junctions[264]. A significant reduction in AChE activity is reported for eels exposed to sublethal concentrations of organophosphorus insecticides and carbamate herbicides already after one hour of exposure[265] [266] [267], thus confirming the suitability of this biomarker for assessing early exposure to neurotoxic compounds.

[260] Ek H., Dave G., Birgersson G., Förlin L., 2003. Acute effects of 2,4,6-trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout (*Oncorhynchus mykiss*). Aq. Ecos. Health. Man. **64**: 415-421.

[261] Reddy G., Chandra S.A. M., Lish J.W., Qualls C.W., 2000. Toxicity of 2,4,6-Trinitrotoluene (TNT) in Hispid Cotton Rats (*Sigmodon hispidus*): Hematological, Biochemical, and Pathological Effects. Int. J. Toxicol. **19** (3): 169-177.

[262] Johnson L.R., Davenport R., Balbach H., Schaeffer D., 1994. Comparative toxicity of trinitrotoluene and aminodinitrotoluenes to *Daphnia magna*, *Dugesia dorotocephala* and sheep erytrocytes. Ecotox Env Saf, **27**: 34-49.

[263] Zitting A, Szumanska G, Nickels J, Savolainen H, 1982. Acute toxic effects of trinitrotoluene on rat brain, liver and kidney: role of radical production. Arch Toxicol, 51: 53-64.

[264] Sturm A., da Silva de Assis H.C., Hansen P.D., 1999. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Mar. Env. Res. **47**: 389-398.

[265] Cèron J.J., Ferrando M.D., Sanche E., Gutierrez-Panizo C. and Andrei-Moliner E., 1996. Effects of diazon exposure on cholinesterase activity in different tissues of European eel (*Anguilla anguilla*). Ecotoxicol. Environ. Saf., **35**: 222-225.

[266] Sancho E., Ceròn J.J. and Ferrando M., 2000. Cholinesterase activity and haematological parameters as biomarkers of sublethal molinate exposure in *Anguilla anguilla*. Ecotoxicol. Environ. Saf. **46**: 81-86.

[267] Intorre L., Soldani G., Cognetti-Varriale A.M., Monni G., Meucci V., Pretti C., 2004. Safety of azamethiphos in eel, seabass and trout. Pharmacol. Res. **49**: 171-176.

6.2.4.1 MATERIALS AND METHODS

The procedures which have been developed in order to prepare the liver microsomes, to analyse the total P450 content and the UDPGT activity as well as the total protein concentrations and the statistical analysis are better specified in par. related to laboratory analyses for yperite effects.

Catalytic assays

The CYP1A and 2B-like enzymes EROD, MROD and BROD activities were measured according to the fluorimetric methods of Lubet, (1985)[268] using a Perkin-Elmer LS50B luminescence spectrofluorimeter. EROD, MROD and BROD assay conditions in the reaction mixture (final volume 2.25 ml) were as follows: pH 7.5, 30°C, in a fluorimeter cuvette containing 50 mM Tris-HCl, 25 mM $MgCl_2$ $6H_2O$, 125 µM NADPH and about 50-100 µl of eel liver microsomal fraction. 7-ethoxyresorufin, 7-methossiresorufin and 7-benzyloxiresorufin (10 µl 0.1 mgml-1 in DMSO), 0.1 was used as the substrate. The reaction started by adding NADPH and the progressive increase in fluorescence was recorded for 4 minutes at λEX=522 nm/λEM=586 nm. The amount of produced resorufin was calculated from a pure resorufin standard calibration curve with a detection limit of 0.05. EROD, MROD and BROD activities were expressed as picomoles of resorufin produced per minute per milligram of total microsomal protein ($pmol\ min^{-1}\ mg\ prot^{-1}$).

NADPH cytochrome c reductase activity measurements were performed according to the method of Livingstone & Farrar (1984)[269] with the following assay conditions: 25 °C, 100mM Tris-HCl (pH 7.6), 20mM KCN, 10mM cytochrome c and 50 µl of liver microsomal suspension. The reaction started by adding 2µM NADH and the progressive decrease in absorbance was recorded for 1 min at a wavelength of 420 nm. The resultant NADPH cyt. c enzyme activity was expressed as nmol min-1mg prot-1. Assays were carried out in triplicate using a Shimadzu UV-160A visible recording spectrometer.

Cholinesterases activity

[268] Lubet R.A., Mayer R.T., Cameron J.W., Nims R.W., Burke M.D., Wolff T. Guengerich F.P., 1985. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. Arch. Biochem. Biophys. **238**: 43-48.

[269] Livingstone D.R. and Farrar S.V., 1984. Tissue and subcellular distribution of enzyme activities of mixed-function oxygenase and benzo[a]pyrene metabolism in the common mussel *Mytilus edulis* L.. Sci. Total. Env. **39**: 209-235.

ChEs were extracted from dorsal muscle homogenates in the proportion 0.1g x 1 ml in ice-cold 20 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mgml-1 Bacitracin, 8 x 10^{-3} TIUml-1 Aprotinin and 1% Triton X-100 (pH 7.6). The tissue was first finely cut with scissors, then homogenized in a Potter-Elvenjem glass/Teflon homogenizer at 2000 rpm and in Ultraturrax and centrifuged at 8400 rpm for 20min at 4°C. The resulting pellet containing cell debris was discarded and the supernatant was immediately analyzed. ChE activity was measured on microplate by the method of Ellman et al. (1961). Assays were performed at 30°C in 0.1 M Na_2PO_4 pH 7.2, and 0.5 mM DTNB, using 1 mM ASCh, as substrate. Activity was measured after incubating for 15 minutes Iso-OMPA (3mM), a selective inhibitor of butyrylcholinesterase (BChE) enzyme, often used to best accomplish the separation of this enzyme from the acetylcholinesterase (AChE).

The increase in absorbance at 405 nm was monitored for 5 min using a 550 Model microplate reader (Bio-Rad). ChE activity was expressed as nmol min^{-1}mg $protein^{-1}$.

6.2.4.2 RESULTS

In vivo study

The experiments show significant results especially on the group tested for 24 h.

The table reports the main results related to the laboratory analyses of Total P450 content, NADPH cyt c red, UDPGT and EROD activities in liver of European eels exposed for 6 and 24 hours to different concentrations of TNT.

	Time	Control	0.5 mg/l	1 mg/l	2.5 mg/l
TOTAL P450	6h	576.02 ± 63.04	567.33 ± 59.69	474.02 ± 41.64	639.48 ± 64.26
nmol/min/mg prot	24h	465.99 ± 88.21	400.42 ± 24.94	455.31 ± 94.02	549.63 ± 34.59
NADPH cyt c red	6h	7.95 ± 1.71	9.18 ± 0.45	9.07 ± 1.87	8.80 ± 1.85
nmol/min/mg prot	24h	7.94 ± 1.98	7.02 ± 1.65	8.75 ± 1.48	10.41 ± 0.52 *
UDPGT	6h	35.46 ± 2.27	34.53 ± 1.08	35.94 ± 4.53	38.86 ± 1.26
nmol/min/mg prot	24h	29.83 ± 1.29	30.01 ± 4.34	38.34 ± 2.15	40.37 ± 4.33
EROD	6h	110.17 ± 10.35	78.24 ± 9.12*	63.52 ± 4.47*	66.97 ± 11.87*
pmol/min/mg prot	24h	97.27 ± 14.34	47.04 ± 13.43*	44.33 ± 12.77*	40.21 ± 9.55*
MROD	6h	4.52 ± 1.50	5.78 ± 1.33	5.34 ± 2.59	3.66 ± 0.84
pmol/min/mg prot	24h	7.13 ± 3.39	2.83 ± 0.22	2.09 ± 0.97*	2.45 ± 0.14*
BROD	6h	4.36 ± 2.02	3.58 ± 1.21	2.20 ± 0.82	3.22 ± 0.53
pmol/min/mg prot	24h	3.01 ± 1.91	2.57 ± 0.17	1.76 ± 0.48	2.10 ± 1.11

* Indicates statistical differences from control ($p<0.05$)

Table 6-19: hepatic P450 enzymes in European eel exposed to TNT. Values are expressed as mean ± s.d. n=4

CYP450 catalytic enzymes show at 6 h at the lowest concentration of 0.5 mg/l a significant modulation only for the EROD activity. An evident significant inhibition of EROD activity was observed also at 24 h (55%) with the same trend starting from 0.5 mg/l. On the opposite, MROD activity was not affected after 6 h of exposure while a significant inhibition was observed at 24 h starting from the lowest exposure concentration of 0.5 mg/l (64%). Variations were not significant at highest concentrations (1 and 2.5 mg/l) (Figure 6–40). A high correlation between EROD and MROD inhibition at 24 h of exposure ($r=0.99$) was observed.

BROD activity was inhibited within 24 h of exposure at different concentrations of TNT but this variation was not significant.

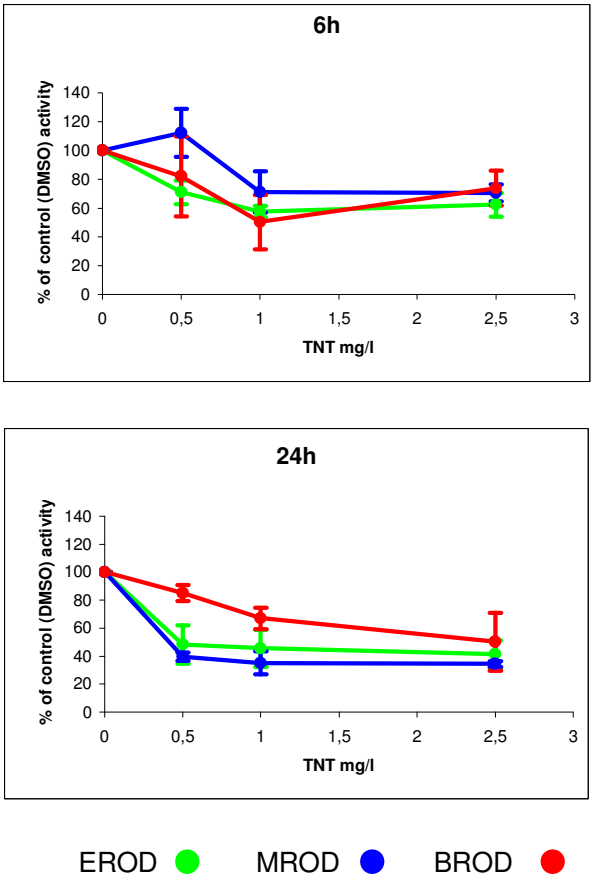


Fig. 6-40 EROD, MROD and BROD activities in liver of European eels treated for 6 and 24 hours with different concentrations of TNT in water. n=4

A dose-dependent slight increase of total P450 was observed after 24 h although no significant differences were measured. A good correlation was observed between total P450 and NADPH cyt c reductase activity at 24 h (r=0.97) which significantly increased (30%) only at the highest concentration 2.5mg/l (Fig 6–41).

The phase II enzyme UDPGT activity tended to increase dose-dependently only at 24 h, although the differences were not significant, nearly 15% of induction was observed; at this time of exposure a good correlation with total P450 was also observed (r=0.73).

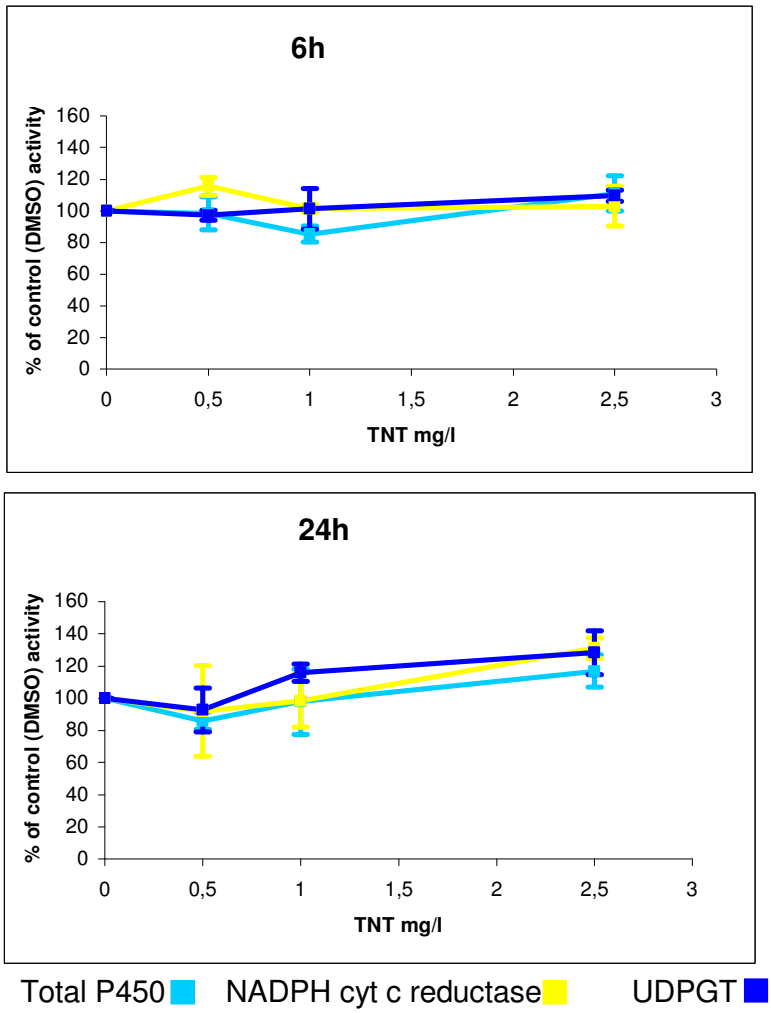


Fig. 6-41 total P450, NADPH cyt c reductase and UDPGT activities in liver of European eels treated for 6 and 24 hours with different concentrations of TNT in water (n=4).

As regards the neurotoxic effects of TNT, it has been interesting to observe stiff and jerkily movements in fishes exposed to 2.5mg/l of TNT.

Regarding ChE versus ASCh activity, no clear neurotoxic effect was evident in muscles except for a significant decrease of activity after 6 h of exposure to 1 mg/L of TNT (Tab 6–20)

	Time	Control	0.5 mg/L	1 mg/L	2.5 mg/L
ChE vs ASCh	6h	158.08 ± 17.38	146.94 ± 20.88	97.61 ± 20.90*	109.37 ± 38.24
nmol/min/mg prot	24h	181.29 ± 43.09	172.79 ± 9.64	172.45 ± 19.98	157.87 ± 61.99

* Indicates statistical differences from control ($p<0.05$)

Table 6-20: ChE vs ASCh activity in muscle of European eel exposed to TNT. Values are expressed as mean ± s.d. (n=4)

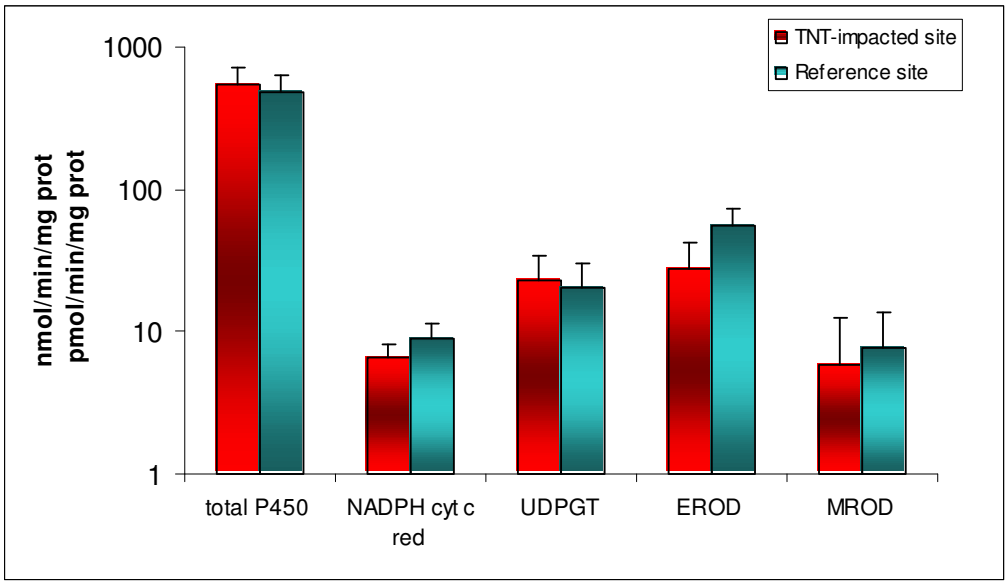
In situ study

In general terms the results confirm those obtained within the *in vivo* experiments. Fishes collected in the TNT-impacted site (Pianosa Island) show an EROD activity significantly lower ($p<0.05$) than the one measured in liver of specimens collected in the reference area (S. Nicola and S. Domino Islands). Total P450 as well as UDPGT activity show higher levels within the TNT-impacted site, despite the difference is not significant. No differences were observed for MROD activity (Tab 6–21 and Fig 6–42.).

	Total P450 nmol/min/mg prot	NADPH cyt c red nmol/min/mg prot	UDPGT nmol/min/mg prot	EROD pmol/min/mg prot	MROD pmol/min/mg prot
TNT-impacted site	551.09 ± 55.88	6.53 ± 1.84	22.88 ± 11.47	27.67 ± 14.47*	5.87 ± 6.60
Reference site	486.71 ± 40.54	8.83 ± 2.41	20.14 ± 9.65	55.72 ± 16.48*	7.75 ± 5.85

* Indicates statistical differences between TNT-impacted and reference site ($p<0.05$)

Table 6-21: hepatic P450 enzymes in European conger from the TNT-impacted site and reference site. Values are expressed as mean ± s.d. (n=18)



* Indicates statistical differences between ($p<0.05$)

Fig. 6-42 hepatic P450 enzymes in European conger from the TNT-impacted site (Pianosa island) and reference site (S. Nicola and S. Domino islands). (n=18)

No differences of ChE versus AsCh activity were detected comparing fishes from the TNT-impacted site with specimens from the reference site (Tab. 6–22).

	ChE vs ASCh nmol/min/mg prot
TNT-impacted site	503.29 ± 67.15
Control site	552.61 ± 78.44

Table 6-22: ChE vs ASCh activity in brains of European conger from the TNT-impacted site and the reference site. Values are expressed as mean ± s.d. (n=18)

6.2.4.3 DISCUSSION

In vivo study

The results of the *in vivo* experiment suggest an active role of the P450 system in the metabolic response performed by fish within 24 h of exposure to TNT, in accordance with other studies carried out on other vertebrate species [270] [271]. A slight response also occurs within 6 h of exposure although it becomes more evident after 24 h at concentrations in the range of LC_{50} values reported for TNT in fish species (0.8 ÷ 3.7 ppm) [272].

The decrease of EROD and MROD activity is in accordance with other studies on freshwater fish species [273] but further studies are needed aimed at verifying the level at which TNT performs its inhibition on CYP1A, either gene expression, protein synthesis, or catalytic level. BROD activity does not seem to be affected by TNT suggesting a low involvement of CYP2B-like in the TNT metabolism/toxicity as reported for other compounds [274].

The results for total P450 content clearly indicate that no modulation of TNT on total P450 occurs in accordance with the statement that total P450 is a less sensitive response to P450 inducers exposure than enzyme activities [275] [276] [277]. NADPH cit c reductase, supposed to be involved in TNT metabolism of mammals as reported by Leung *et al.*, (1995) and Lingyuan *et al.*, (1989)[278],

[270] Leung K.H., Yao M., Stearns R., Chiu S.-H.L., 1995. Mechanism of bioactivation and covalent binding of 2,4,6-trinitrotoluene. Chem Biol Inter, **97**: 37-51.

[271] Johnson M.S., Vodela J.K., Reddy G., Holladay S.D., 2000. Fate and the biochemical effects of 2,4,6-trinitrotoluene exposure to tiger salamanders (*Ambistoma tigrinum*). Ecotox Env Saf, **46**: 186-191.

[272] Talmage S.S., Opresko D.M., Maxwell C.J., Welsh C.J.E., Cretella F,M,, Reno P.H., Daniel F.B., 1999. Nitroaromatic munition compounds: environmental effects and screening values. Rev Env Cont Tox **, 161**: 1-156.

[273] Ek H., Dave G., Birgersson G., Förlin L., 2003. Acute effects of 2,4,6-trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout (*Oncorhynchus mykiss)*. Aq. Ecos. Health. Man. **64**: 415-421.

[274] Fent K., Woodin B.R., Stegeman J.J, 1998. Effects of triphenyltin and other organotins on hepatic monooxygenase system in fish. Comp. Biochem. Physiol. C., **121**: 277-288.

[275] Bucheli D.T. and Fent K., 1995. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystem. Env. Sci. Tec. **25**(3): 201-268.

[276] Ahmad I., Pacheco M., Santos M.A., 2004. Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbor water exposure. Ecotoxicol. Environ. Saf. **57** (3):290-302.

[277] Fent K., Stegeman J.J., 1991. Effects of tributyltin chloride in vitro on the hepatic microsomal mono-oxygenase system in the fish *Stenotomus chrysops*. Aquat.Toxicol. **20**:159-168.

[278] Kong L., Jiang Q., Qu Q., 1989. Formation of superoxide radical and hydrogen peroxide enhanced by trinitrotoluene in rat liver, brain, kidney and testicle *in vitro* and monkey liver *in vivo*. Biomed. Environ. Sci. **2**: 72-77.

shows a slight dose-dependent increase after 24 h. Nevertheless values are significantly higher, when compared to the controls, only for specimens exposed to the highest concentration of TNT (2.5 mg/L) thus indicating a low susceptibility of this enzyme to TNT as well as to other classical xenobiotics inducers of P450 system[279].

Concerning the phase II enzyme UDPGT, an increase is registered only after 24 h of exposure thus suggesting a possible delay in the enzyme's response compared to EROD. The hypothesis is consistent with the findings of a research performed by Zitting *et al.*, (1982)[280] in rat hepatic UDPGT activity which resulted significantly higher than controls only after 48 h of TNT exposure.

Fishes exposed to 2.5mg/l of TNT showed a clear loss of motor capacity after 20 hours of exposure. This observation suggests a potential toxic effect of the compound as already reported in other fish species exposed to TNT[281]. However, the results of ChE activity obtained so far seem to confirm that TNT does not have an anticholinesterase mechanism of action and consequently ChE cannot be considered a good marker of exposure for this pollutant. Several cases are reported in literature where behavioural alterations, similar to the ones observed for TNT, occur without cholinesterase inhibition [282].

In situ study

The results of the field study highlight the presence of a source of contamination, as confirmed by the inhibition of EROD activity in the collected specimens. Moreover, the results seem to validate the suitability of the selected multiple biochemical tools in biomonitoring and assessing the environmental risk of ammunition dumping sites. No inhibition of total P450 content, UDPGT and MROD activity were observed in agreement with data obtained from the *in vivo* study.

[279] Van der Oost R., Beyer J., Vermeulen N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Env., Tox., Pharm. **13**: 57-149.

[280] Zitting A, Szumanska G, Nickels J, Savolainen H, 1982. Acute toxic effects of trinitrotoluene on rat brain, liver and kidney: role of radical production. Arch Toxicol, 51: 53-64.

[281] Smock L.A., Stonuburner D.L., Clark J.R., 1976. The toxic effects of trinitrotoluene (TNT) and its primary degradation products on two species of algae and the fathead minnow. Water Research, **10**: 537-543.

[282] Beauvais S.L., Jones S.B. Parris J.T., Brewer S.K. and Little E.E., 2001. Cholinergic and behavioural neurotoxicity of carbaryl and cadmium to larval rainbow trout (*oncorhynchus mykiss)*. Ecotoxicol. Environ. Saf. **49**: 84-90.

Regarding MROD activity, studies carried out on the European eel CYP1A assume the presence of two forms of CYP1A genes in eel species which might be specific for this species whereas their presence should still be verified in species genetically close to eels but not diadromuous, like European conger[283] [284]. This finding would support our poorly significant MROD results for Conger thus suggesting a scarce suitability of this enzyme as a biomarker of TNT exposure.

The results of this study clearly indicate P450 enzymes as suitable tools for monitoring and assessing the biological impact of conventional dumped ammunitions in marine fish species. Moreover, the inhibition of P450 enzymes, as observed in specimens collected around Pianosa island, may determine a further susceptibility of the organism to other pollutants, as the normal functioning of the system represents a general defence of the organism aimed at protecting the cells from the intracellular accumulation of natural, endogenous toxic substances and xenobiotics.

No alteration was observed in ChE activities of fishes, in agreement with data obtained from the *in vivo* study.

6.2.5 Stress Index: the Heat Shock Protein (Hsp70) Assay

The coelomic cavity of sea urchins contains free cells, generically called coelomocytes. Due to their capability to respond to injuries, host invasion and cytotoxic agents, coelomocytes are considered to be immune effectors of the sea urchin[285] [286] [287] [288].

Many studies have described how coelomocytes respond to both environmental and experimental challenge (*in vivo* and *in vitro* experiments), showing a high suitability of these cells as “bio-indicators”. In fact, coelomocytes are able to respond to different kinds of stress both at cellular and at biochemical levels,

[283] Schlezinger J.J., Stegeman J.J., 2000. Induction of cytochrome P450 1A in the American Eel by model halogenated and non-halogenated aryl hydrocarbon receptor agonists. Aquat. Tox. **50**: 375-386.

[284] Mahata S.C., Mitsuo R., Aoki J.Y., Kato H., Itakura T., 2003. Two forms of cytochrome P450 cDNA from 3-methylcholantrene treated European eel *Anguilla anguilla*. Fish. Sci. **69**: 615-624.

[285] Smith V.J. 1981. The Echinoderms. In: Ratcliffe NA, Rowley AT (eds) Invertebrate Blood Cells. Vol **2**. Academic Press, London.

[286] Coffaro K.A., Hinegardner, R.Y. 1977. Immune response in the sea urchin *Lytechinus pictus*. Science **197**: 1389-1390.

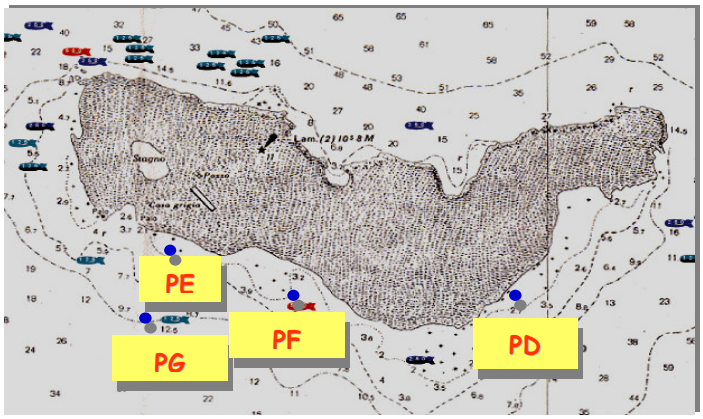
[287] Hilgard H.R., Phillips J.H. 1968. Sea urchin response to foreign substances. Science **161**: 1243-1245.

[288] Smith L.C., Davidson E.H., 1992. The echinoid immune system and the phylogenetic occurrence of immune mechanisms in deuterostomes. Immunol Today **13**: 356-362.

with variations in coelomocytes subpopulations and increase in the expression of a few stress proteins such as Hsp70, p53, p63, 14-3-3, AchE[289 290 291 292].

6.2.5.1 MATERIALS AND METHODS

The Heat Shock Protein (Hsp70) Assay has been applied on coelomocytes of specimen of sea urchin (*Paracentrotus lividus*) collected around the Pianosa Island as well as in a reference site within the same archipelago (Caprara Island).



PE site: 20 samples collected at 3 m deep;

PF site: 10 samples collected at 3 m deep;

PG site: 10 samples collected at 10 m deep;

PD site: 20 samples collected at 3 m deep (near a ship-wreck)

Fig. 6-43 map showing the sampling sites around Pianosa Island

The total number of samples collected was 83; coelomocytes pellets from each of the 83 specimens were divided into 5 tubes. Pellets stored in liquid N_2 were then transferred to the CNR Institute Biomedicina e Immunologia Molecolare "Alberto Monroy" in Palermo where the following procedure was performed:

1. lysis of pellets in 500µl of lysis buffer pH 7.5 (20 mM Tris, 2 mM EDTA, 1% NP-40, 15% glycerol, 2 mM DTT) supplemented with a cocktail of protease inhibitors;
2. determination of pellets protein content by the Bradford method (Bio-Rad Protein Assay);

[289] Matranga V., Toia G., Bonaventura R., Mùller W.E.G., 2000. Cellular and biochemical responses to environmental and experimentally induced stress in sea urchin coelomocytes. Cell Stress and Chaperones **5** (2): 113-120.

[290] Matranga V., Bonaventura R., Di Bella G., 2002a. HSP70 as a stress marker of sea urchin coelomocytes in short term cultures. Cellular and Molecular Biology **48** (4): 345-349.

[291] Matranga V., Pinsino A., Celi M., Di Bella G., Natoli A., 2006. Impacts of UV-B radiation on short term cultures of sea urchin coelomocytes. Marine Biology **149**: 25-34.

[292] Angelini C., Amaroli A., Falugi C., Di Bella G., Matranga V., 2003. Acetylcholinesterase activity is affected by stress conditions in *Paracentrotus lividus* coelomocytes. Mar Biol **143**: 623-628.

3. separation of Coelomocytes lysates (15 µg) on 7,5% SDS-PAGE under reducing conditions;
4. transfer of proteins separated by SDS-polyacrylamide minigels to nitrocellulose paper;
5. immune reaction with Hsp70: it was used an anti-bovine brain 70 kDa heat shock protein monoclonal antibody (Hsp70 McAb) (SIGMA) diluted 1 to 5000. The second antibody was a peroxidase- conjugated anti-mouse IgG (AMERSHAM), diluted 1 to 5000);
6. Super Signal (PIERCE) was used for detection of bands.
7. Quantification of band intensities by scanning, with a Bio-Rad imaging densitometer (Model Gel Doc 1000) equipped with an analysis programme automatic integrator (multiAnalyst, version 1.1).

Ten samples were already degraded before the analyses, probably due to a previous melting during the storage period.

6.2.5.2 RESULTS

Results on the levels of Hsp70, obtained by immunoblotting on 39 different samples are shown in Fig. 6–44.

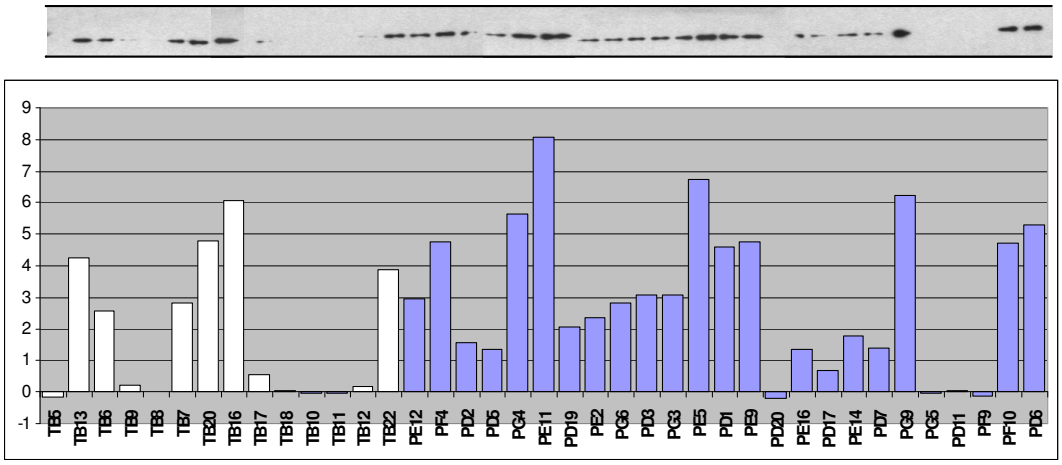


Fig. 6-44 Hsp70 expression in 39 samples of coelomocytes (14 samples around Caprara Island, reference site, and 25 samples around Pianosa Island)

An increase in the Hsp70 levels expressed in sea urchins from the polluted area (25 samples around Pianosa Island) versus those from the reference site (14 samples around Caprara Island) was found. However, a certain variability is

found both in basal and stress levels of Hsp70 among individuals, as estimated by a semi-quantitative analysis. The graphical representation, shown in Table 1, summarizes the results obtained by Western blot analysis of all the 73 samples. The matrix contains in the vertical row on the left 53 different samples collected in the impacted area (Pianosa) and in the top horizontal line 20 different samples collected in the reference site (Caprara). Each intersection is reporting the ratio between the band intensity of impacted versus the one of control sample. The values of samples with Hsp70 levels higher than controls is shown in red. In yellow, those with equal or lower levels. In white samples not determined. The mean value obtained for each experimental impacted sample is reported within the last column on the right of the table.

	TB5	TB6	TB7	TB8	TB10	TB9	TB11	TB12	TB13	TB16	TB22	TB20	TB18	TB17	TB1	TB14	TB15	TB18	TB19	TB23	
PG4	84	2,2	2	47,5	76	25,3	94,9	23	1,4	1	1,5	1,3	759	9,26	ND	ND	ND	ND	ND	ND	80,59714
PG6	40	1	0,93	23	36,3	12,1	45,25	10,96	0,7	0,4	0,7	0,6	362	4,4	ND	ND	ND	ND	ND	ND	38,45286
PG3	51	1,3	1,2	28	45,5	15,1	56,75	13,75	0,8	0,6	0,9	0,7	454	5,54	ND	ND	ND	ND	ND	ND	48,22429
PG9	94	2,5	2,2	53	84,3	28	105	25,5	1,5	1,2	1,6	1,44	841	10,8	ND	ND	ND	ND	ND	ND	89,43143
PG5	0	0	0	0,2	0	0,1	0,4	0	0	0	0	0	3	0	ND	ND	ND	ND	ND	ND	0,264286
PG1	0	0	0	0	0	0	0	0	ND	ND	0	ND	ND	0	0	0	0	0	0	0	0
PG2	246	0,7	0,5	8,2	247	1,7	247	4,3	ND	ND	0,7	ND	ND	30,9	9,46	0,75	24,6	1,8	247	248	82,41313
PG8	374	1,1	0,7	12,5	375	2,5	375	6,5	ND	ND	1	ND	ND	46,75	14,4	1,1	37,4	2,8	375	376	125,1094
PG10	0	0	0	0	0	0	0	0	ND	ND	0	ND	ND	0	0	0	0	0	0	0	0
PD5	25	0,65	0,6	14	22,4	7,47	28	6,78	2,43	0	0,4	0,4	224	2,73	ND	ND	ND	ND	ND	ND	23,91857
PD19	31	0,8	0,7	17	27,6	9,2	34,4	8,33	0,5	0	0,5	0,5	275	3,35	ND	ND	ND	ND	ND	ND	29,20571
PD3	46	1,2	1	26	41,5	13,8	51,75	12,54	0,8	0,6	0,8	0,7	414	5	ND	ND	ND	ND	ND	ND	43,97786
PD1	70	1,8	1,6	39	62,3	20,83	78,12	18,94	1,15	0,9	1,2	1,1	625	7,6	ND	ND	ND	ND	ND	ND	66,39571
PD20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	0
PD17	10	0,3	0,2	5,5	8,9	2,9	11	2,7	0	0	0	0,15	88	1	ND	ND	ND	ND	ND	ND	9,332143
PD7	22	0,6	0,5	12	19,7	6,5	24,5	5,94	0,4	0,3	0,4	0,3	196	2,4	ND	ND	ND	ND	ND	ND	20,82429
PD11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	0
PD6	81	2,1	1,9	46	73,1	24,3	91,2	22,12	1,33	1	1,4	1,25	730	8,9	ND	ND	ND	ND	ND	ND	77,54286
PD2	25	0,6	0,6	14	22,5	7,5	28,12	6,82	0,4	0,3	0,4	0,4	225	2,7	ND	ND	ND	ND	ND	ND	23,88143
PD4	214	1,6	1	18,6	559	3,8	559	9,75	ND	ND	1,5	ND	ND	69,75	21,4	1,7	55,8	4,2	559	560	165,0063
PD8	285	2,2	1,4	24,7	743	5	742	13	ND	ND	2	ND	ND	92,75	28,5	2,3	74,1	5,5	743	745	219,3406
PD9	203	1,5	1	17,6	530	3,6	529	9,22	ND	ND	1,5	ND	ND	66,1	20,3	1,6	52,9	4	530	531	156,395
PD10	226	1,7	1,1	19,6	591	4	591	10,3	ND	ND	1,6	ND	ND	73,8	22,6	1,8	59	4,4	591	593	174,4938
PD12	545	4,1	2,7	47,3	1422	9,6	1422	24,8	ND	ND	3,9	ND	ND	177,5	54,5	4,3	142	10,6	1423	1426	419,9563
PD13	614	4,6	3	53,2	1601	10,9	1601	27,9	ND	ND	4,43	ND	ND	195	61,4	4,9	160	12	1602	1607	472,6456
PD14	568	4,3	2,8	49,2	1480	10	1480	25,8	ND	ND	4,1	ND	ND	180	56,8	4,5	148	11	1481	1485	436,9063
PD15	347	2,6	1,72	30,1	906	6,2	906	15,8	ND	ND	2,5	ND	ND	113	34,7	2,8	90,5	6,77	906	909	267,5431
PD16	359	2,7	1,8	31,1	936	6,3	936	16,3	ND	ND	2,6	ND	ND	117	35,9	2,8	93,5	6,96	936	939	276,435
PD18	233	1,7	1,15	20,2	608	4,1	607	10,5	ND	ND	1,7	ND	ND	76	23,3	1,85	60,7	4,5	608	609	179,4188
PE1	84,7	2,6	0	1,1	84,7	84,7	8,38	5,1	ND	ND	ND	ND	3,8	1,5	84,7	0,76	3,4	ND	0,8	0	24,416
PE3	146,7	4,4	1,3	2	146,7	146,7	14,5	8,8	ND	ND	ND	ND	6,5	2,6	146,7	1,3	6	ND	1,3	1,5	42,46667
PE4	158,3	4,8	1,4	2,1	158,3	158,3	15,7	9,55	ND	ND	ND	ND	7	3	158,3	1,4	6,5	ND	1,4	1,6	45,84333
PE8	180	5,5	1,6	2,4	180	180	17,9	10,8	ND	ND	ND	ND	8	3,2	180	1,6	7,3	ND	1,6	1,9	52,12
PE13	64,3	2	0,5	0,8	64,3	64,3	6,4	3,9	ND	ND	ND	ND	2,9	1,2	64,3	0,6	2,6	ND	0,6	0,7	18,62667
PE15	142,7	4,2	1,2	2	142,7	142,7	14,3	8,6	ND	ND	ND	ND	6,4	2,5	142,7	1,3	5,8	ND	1,3	1,5	41,32667
PE17	9	0	0	0	9	9	0	0,5	ND	ND	ND	ND	0	0	9	0	0	ND	0	0	2,433333
PE19	99,3	3	0,8	1,3	99,3	99,3	9,83	6	ND	ND	ND	ND	4,4	1,8	99,3	0,9	4	ND	1	1	28,74867
PE12	42	1	1	24	37,9	12,6	47,4	11,5	0,7	0,5	0,74	0,6	379	4,6	ND	ND	ND	ND	ND	ND	40,25286
PE5	102	2,7	2,4	57	92	30,6	114,75	27,82	1,7	1,3	1,8	1,6	918	11,2	ND	ND	ND	ND	ND	ND	97,49071
PE9	65	1,7	1,5	36	58	19,3	72,4	17,54	1	0,8	1,1	1	579	7,1	ND	ND	ND	ND	ND	ND	61,53143
PE16	18	0,5	0,4	9,9	15,9	5,3	19,9	4,82	0,3	0,2	0,3	0,3	159	2	ND	ND	ND	ND	ND	ND	16,91571
PE14	29	0,8	0,7	16	26,2	8,7	32,8	7,94	0,5	0,4	0,5	0,44	262	3,2	ND	ND	ND	ND	ND	ND	27,79857
PE2	35	0,9	0,9	19,7	31,6	10,5	39,5	9,57	0,6	0,4	0,6	0,5	316	3,85	ND	ND	ND	ND	ND	ND	33,54429
PE20	0	0	0	0	0	0	0	0	ND	ND	ND	ND	0	0	0	0	0	ND	0	0	0
PF2	109	3,3	0,97	1,45	109	109	10,8	6,6	ND	ND	ND	ND	4,8	1,9	109	1	4,4	ND	1	1,1	31,55467
PF3	0	0	0	0	0	0	0	0	ND	ND	ND	ND	0	0	0	0	0	ND	0	0	0
PF5	0,7	0	0	0	0,7	0,7	0	0	ND	ND	ND	ND	0	0	0,7	0	0	ND	0	0	0,186667
PF7	0	0	0	0	0	0	0	0	ND	ND	ND	ND	0	0	0	0	0	ND	0	0	0
PF8	0	0	0	0	0	0	0	0	ND	ND	ND	ND	0	0	0	0	0	ND	0	0	0
PF9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	ND	ND	ND	ND	ND	ND	0
PF10	72	1,9	1,7	40	64,7	21,5	80,6	19,5	1,2	0,9	1,3	1,1	645	7,9	ND	ND	ND	ND	ND	ND	68,52143
PF4	70	1,8	1,6	39	63	20,9	78,6	19,06	1,16	0,9	1,2	1,1	629	7,7	ND	ND	ND	ND	ND	ND	66,78714
	119,6288	1,633654	0,966731	17,54327	228,8865	26,24808	215,926	9,791346	0,77375	0,4875	1,180789	0,645	240,1789	26,29769	49,21286	1,402143	37,08929	5,323571	357,5	358,475	

Table 6-23; Hsp70 expression. The values represent the ratio between the Hsp70 levels in samples collected in impacted area versus those measured in samples collected in reference site. In red the values higher than 1; in yellow the values equal or lower than 1.

Finally, Fig. 6–45 shows a diagrammatic distribution of increase in Hsp70 expression for samples collected within the impacted areas, named PG, PD, PE and PF, expressed as fold increase over controls. Specifically, an average 51-fold increase for the PG site, a 153-fold increase for the PD site, a 39-fold increase for PE site and a 21-fold increase for the PF site were found.

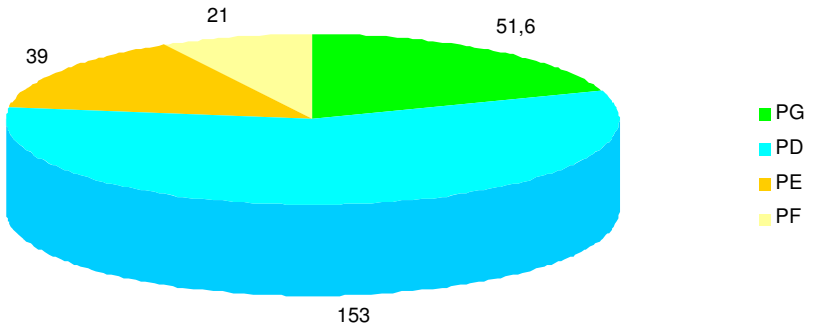


Fig. 6-45 distribution of increase in Hsp70 expression of samples collected in the impacted areas, expressed as fold increase over controls.

6.2.6 Discussion of Laboratory Results

The absence of detectable traces of TNT and its biodegradation products in fish tissues confirms their low bioaccumulation and bioconcentration capability whereas the high recovery of the standards confirms the applicability of the GC-ECD/GCMS assay.

Laboratory results show significant differences between the values of stress indexes observed in samples collected within the study area versus those coming from the other islands of Tremiti archipelago, chosen as the reference site. In particular, a significant increase of protein Hsp 70 in coelomocytes of *P. lividus* and a reduction of hepatic P450 enzyme activities (in particular EROD activity) in *C. conger* was observed. A similar pattern was registered in specimens of *Anguilla anguilla* exposed *in vivo* to different concentrations of TNT.

In conclusion, the alterations registered in all the biological parameters examined within the different species seem to suggest that the multiple stress-response approach is a suitable tool for environmental monitoring programmes.

The current research has also made available new data regarding biomarkers of exposure and effect in European conger which may represent a potentially very useful species for future eco-toxicological studies within the marine environment, as already confirmed in the CWAs study of the present project. Finally, the suitability of Hsp70 and hepatic P450 enzyme activities as valuable markers of stress is reinforced, highlighting their possible use in future similar studies.

7 The Best Available Technologies to Clean-up the CW Dumping Sites at Sea

The results obtained have provided us with a worrying picture of the situation in our seas concerning both the extension of pollution and the ecological impact of dumped ordnance. A possible solution to the problem is the cleaning up of the areas affected by CW dumping. Nevertheless, there are however evident difficulties in carrying out such an operation. Over the last years the scientific community has posed the question as to the best solution to the problem. The possible solutions include leaving the munitions on the sea-bed undisturbed, attempting to recover and treat them in some way in order to render them completely harmless, or examining possible ways in which the material can be rendered completely harmless *in situ*. The answer to this question in the documentation reviewed is unanimous. It states that munitions dumping sites should remain undisturbed. A literature review made by the Imperial College of London[293] reports all the bibliographical references supporting this argument, already reported in par. 5.7(Studies on Environmental Threats of CWs Dumped at Sea). This solution is recommended because at present the chemical weapons dumped at sea seem to represent no acute danger either to human-beings or to the marine environment. Moreover, it should be taken into account that clean up operations could increase the risk of a massive release of the toxic compounds into the marine environment. This could be caused by the displacement of rusted munitions during the proceedings. Despite this, we believe that it would be advantageous to carry out research in order to assess the best available technologies for the operation, in terms of money, time and safety for both man and the environment. Only after the data from a pilot clean up activity are available would it be fair to make a final decision on whether to remove the chemical weapons from the seafloor or to leave them where they are.

Below some of the Best Available Technologies to identify and to remove the munitions lying on the seafloor are examined.

[293] Beddington J., Kinloch A.J., 2005. *Munitions Dumped at Sea: A literature Review.* Imperial College London Consultants. 90 pp. www.imperial-consultants.co.uk and www.mod.uk/NR/rdonlyres/77CEDBCA-813A-4A6C-8E59-16B9E260E27A/0/ic_munitions_seabed_rep.pdf

7.1 Technologies available to identify ordnance on the seafloor

The methodology and instruments employed during the survey undertaken in July 1999, during the A.C.A.B. project (par. 5.7.1) seem to be able to identify metal targets on the seafloor very effectively. The side scan sonar used with a magnetometer are particularly effective. Moreover, the latest versions of these instruments can also find targets with a length of less than 1 meter, this is the case of several types of artillery bombs which have an average length of about 50 cm.

However, the use of these current, non-specific techniques to locate dumped weapons often results in a high false-positive rate. The use of chemical detectors could be another useful means of identifying ordnance dumped at sea. The compounds contained in weapons (e.g. TNT and yperite) are man made molecules not produced by any natural source and can therefore be detected by chemical detectors. For this task many different sensitive and analytical techniques could be used, which are based principally on Ion Mobility Spectrometry (IMS), Mass Spectrometry (MS), Flame photometry (FPD), Gas Chromatography used with MS, FPD or IMS; Surface Acoustic Waves (SAW), X-Ray techniques and Gamma Spectrometry. Chemical detection of explosives in a marine environment is an extremely challenging problem. For example, an explosive chemical signature emanating from a source can rapidly disperse in the environment, diluting the signature to levels that are difficult to detect. This dispersion is often highly directional, making it necessary for the sensor to be positioned in the chemical plume emanating from the target. Moreover, because of the low solubility in water of explosive compounds and chemical agents such as yperite, their concentration in the water is likely to be very low. This increases the sensitivity requirements of chemical sensors designed to detect the signatures.

The traditional way to check for contamination from CW agents on the sea bottom is to take samples and analyse them in the laboratory. Many samples have to be taken and expensive refrigeration and transportation provisions are mandatory. The analytical procedure requires an established and validated operating protocol that takes into account the expensive, time consuming and sophisticated techniques involved. An alternative way of looking for dumped CWs could be based on analytical instrumentation directly installed in special submarines, such as the Remotely Operated Vehicle (like the one used during A.C.A.B. survey) or the Autonomous Underwater Vehicle (AUV), which are

equipped with specific sampling apparatus. The AUVs are vehicles that are able to move in water without a wire using a remote control. It is possible to reach the expected dumping site with these underwater vehicles and analyse instantly even very low traces of different chemicals dispersed in the water. It is also clear that only very special analytical instruments can be used for this particular task.

The University of Hamburg (Germany) together with Bruker Daltonics have developed a special ROV equipped with a Gas Chromatography used with a Mass Spectometry (Bruker Daltonics GC-MS EM640) (personnel communication by Luca Pinciarelli of Bruker Daltonics). The sampling apparatus is based on a silicone membrane sampler. It is possible to sample and enrich even very low traces of different chemicals dispersed in the water (part per million) with the sampling probe. These sampled chemicals are then desorbed directly in the capillary column of the instrument where they are separated and injected into the mass spectrometer for detection. All data can be transmitted instantly to the control room on the ship.

The USA Office of Naval Research (ONR) sponsored the Chemical Sensing in the Marine Environment (CSME) program. This research aimed to solve the problem of locating underwater Unexploded Ordnance (UXO) using novel techniques to detect and locate them. Specific emphasis is directed towards the development and refinement of chemical sensors mounted on a AUV. This system seems to be able to map and trace chemical plumes coming from rusted ordnance lying on the seafloor [294] [295]. The electrical and mechanical integration of the SPAWAR Systems Center REMUS AUV (Fig. 7–1) with the Nomadics, Inc. SeaDog and SeaPup analyte sensors as well as the New Mexico State University (NMSU) analyte sensor have been tested using explosive source simulators. The SeaPup sensor was the best performer, and repeatedly mapped analyte plumes of 50-58 ppb TNT to 75-100 m downstream from the source.

[294] Arrieta R., Granger B., Djapic V., 2004. *Development of Chemical sensing and Plume tracing of UXO using Unmanned Undersea Vehicle*. In acta of "Sixth International Symposium on Technology and the Mine Problem", Monterey California.

[295] *Dock M., Sikes J., Fisher M., 2004. Underwater Explosives Detection Using a Chemical Sensing Method.* In acta of "Sixth International Symposium on Technology and the Mine Problem", Monterey California.

Fig 7–1 Autonomous Underwater Vehicle (AUV) REMUS [293]

In addition to nitroaromatic explosives (TNT and its degradation products), the chemical sensors tested during the CSME program can detect RDX (cyclonite or hexogen), HMX (Octogen) and PETN (Pentaerythritoltetranitrate). SeaDog and SeaPup sensors utilise amplifying, fluorescent, polymer technology. The fluorescence is greatly reduced when exposed to target explosive compounds. The instrument is able to detect chemical plumes and, with adaptive mission programming, trace autonomously the plume to its origin and discover the source location (fig. 7–2).

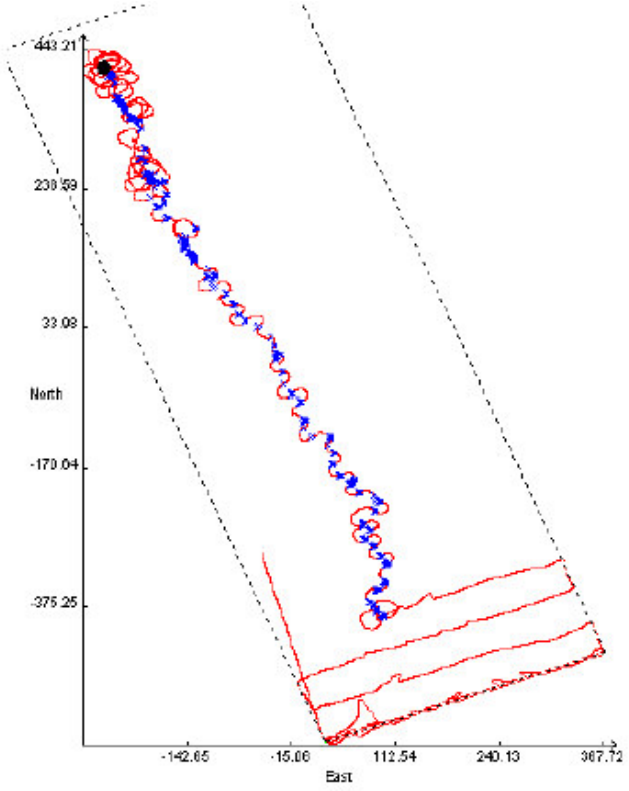


Fig 7–2 REMUS trajectory (in red). It is possible to observe as the sensor detects the explosive simulator compound the AUV change its programmed course and follow the tracks detected until it arrives at the source. Blue X's indicate chemical detection locations [293]

The validation of possible ordnance sightings can be obtained through a video-camera or a sonar sensor mounted on an AUV.

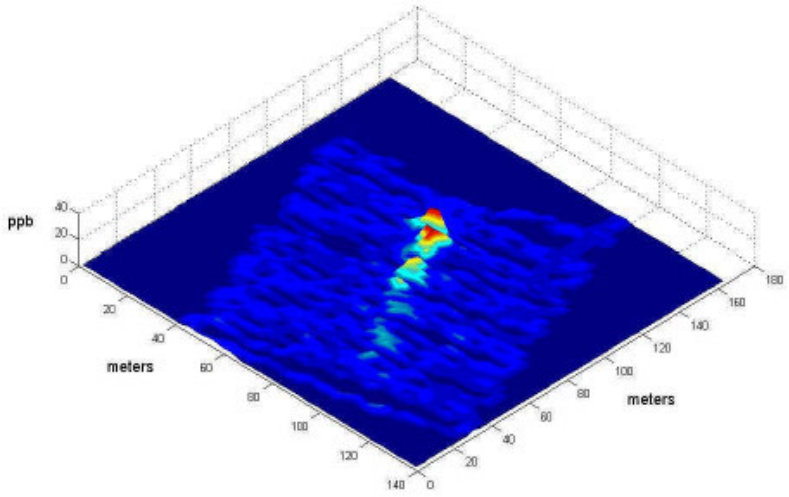


Fig 7–3 TNT plume map [293]

The researchers conducting the experiment underline that the technology is not fully mature yet. In order to validate this technology, actual concentrations of explosive residues around individual UXO items or UXO dumping sites must be obtained.

Rodacy *et al.* [296]developed a procedure that allowed them to detect in September 1999 explosive compounds in water and sediment near conventional munitions dumped in Bedford Basin, Halifax, Nova Scotia, after WW I and II.

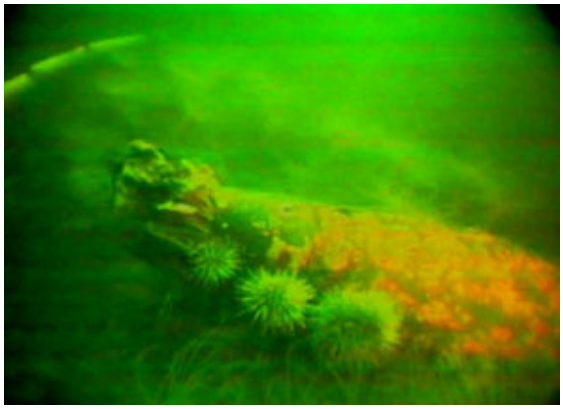


Fig 7–4 Ib bomb in Halifax harbour [294]

[296] Rodacy P.J., Walzer P.K., Reber S.D., Phelan J., 2004. *Esplosive Detection in the Marine Environment Using Ion Mobility Spectroscopy*. In acta of "Sixth International Symposium on Technology and the Mine Problem", Monterey California.

The process of detecting explosive signatures in water included three basic steps. The first step involved sampling the water and sediment near the target. The second step involved separating and concentrating the explosive molecules from the sample and finally, the third step involved transferring the explosive analyte to a detector for processing. The analyte concentration is made of Solid Phase Microextraction (SPME), that is an absorbent coated fibre capable of removing and concentrating the compounds from the sample. The SPME fibre is then inserted directly into the analyser - an ion mobility spectrometer (IMS). This technique allowed the researchers to detect explosive concentration at ppb level. For example: 59 water samples were analysed and 34 samples (58%) produced detectable explosive signatures, 27 sediment samples were analysed and 26 samples (96%) produced detectable explosive signals. Moreover TNT metabolites were also present. The concentrations were observed to decrease with increasing distance from the target.

7.2 Hypothesis for remediation

A Synopsis published in June 1999 under the auspices of the Dr. A. H. Heineken Foundation for the Environment (NL) [297], provides useful indications regarding some possible remediation hypothesis. These solutions could also be considered for the chemical ordnance dumped in the Southern Adriatic Sea. Among the different options, two technologies in particular, which are described below, could be considered for the clean up activities of CWs in the Adriatic.

- Capping
- Recovery and disposal

The two techniques are described below. We have considered the indications reported in the synopsis as well as other more updated information obtained during the bibliographical research.

7.2.1 Capping

This option aims to completely cover the war material in order to avoid the leakage of its contents into the marine environment. The advantages of this type of technology are described below:

[297] Duursma E. K., 1999. *Dumped Chemical Weapons in the Sea: Options*. Synopsis published under the auspices of the Dr. A.H. Heineken Foundation for the Environment, Amsterdam (Netherlands).

- isolates the chemical agents preventing their dispersion, especially if the capping material has a low permeability to fluids
- protects the buried material from any contact with fishing equipment or other human activities
- reduces the corrosion rate of the ordnance
- facilitates the transformation of chemical agents if the capping material is added with reacting compounds.

Capping of wrecks

The capping of wrecks loaded with chemical weapons can be carried out *in situ*. The capping material should be made of shattered rocks. A preliminary survey is necessary in order to evaluate: the nature and morphology of the sea bed; size, shape and position of the wreck; the state of corrosion of both the wreck and the ordnance. Data, such as the position and the size of the wreck are extremely important in order to decide the most effective way of intervening and the amount of material necessary for the capping activities. It has been estimated that the minimum amount of material necessary to cover a "*Liberty*" ship (model generally used for dumping war material) is nearly 250,000 tons. The quantities may be reduced if the wreck is partially covered by sediments or if it is standing in a tilted position (Fig. 7–5).

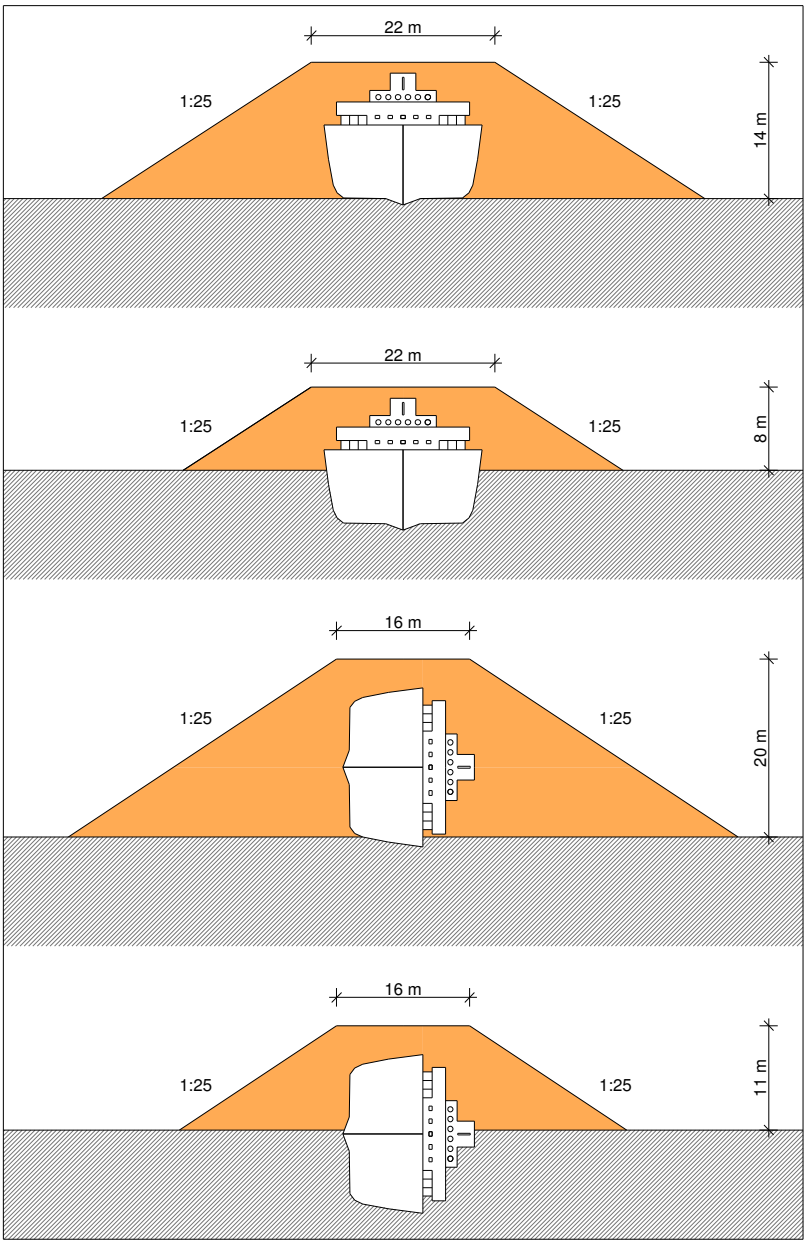


Fig. 7–5 Different capping conditions of a wreck loaded with chemical weapons, depending on wreck position.

The capping material can be emitted by means of a tube with a diameter of 0.5-1 m, which is able to transfer the material from the support vessel to the sea bed. The end of the tube is connected to a *Heavy Working Class R.O.V.* (*Remotely Operative Vehicle*) specially equipped with underwater cameras and independent engines (Fig. 7–6).

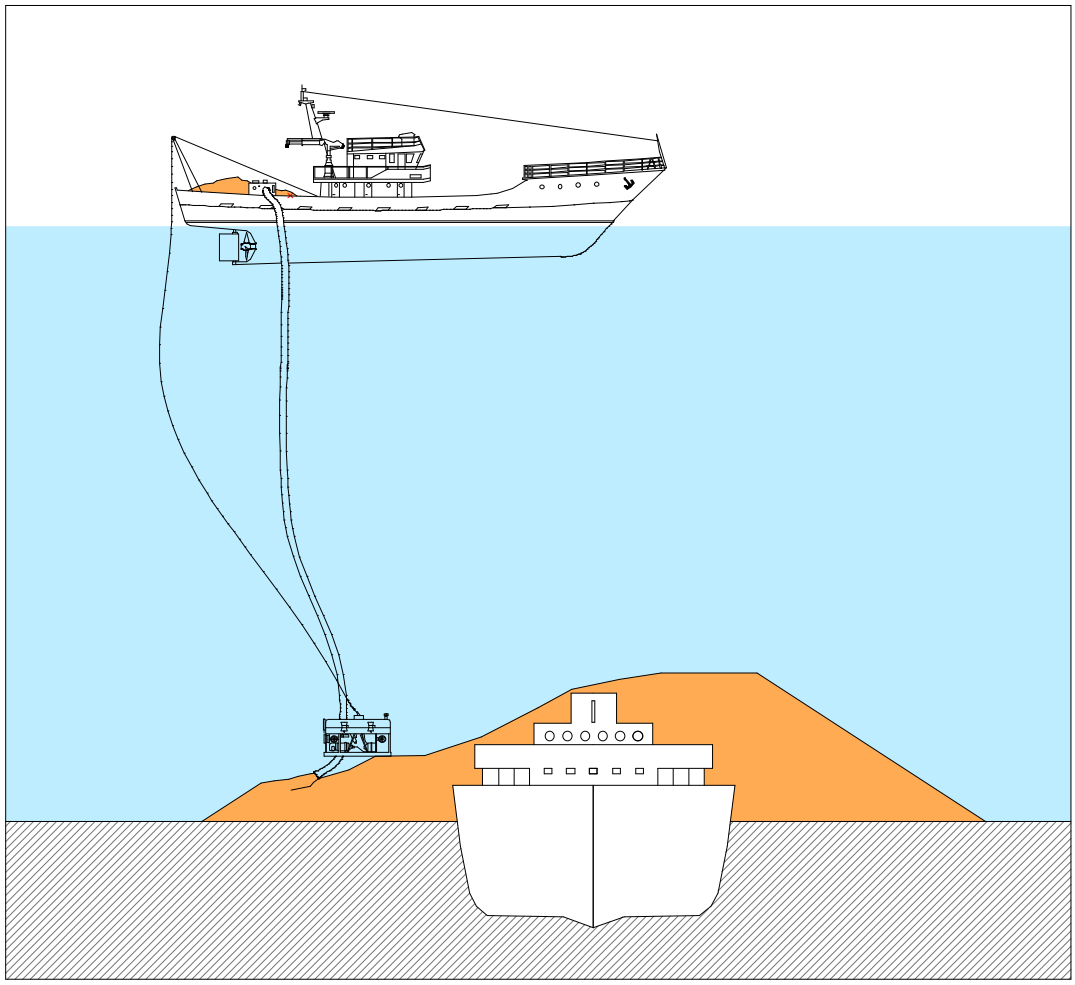


Fig 7–6 Capping of a wreck by a R.O.V.

The granulometry of the external layer of the capping material must be able to resist to the erosive action of external agents and fishing gears. Moreover, in order to avoid the collapse of this coarse material, the presence of internal layers, with a decreasing granulometry towards the sea bottom, is necessary. The granulometric ratio generally employed between two intermediate consecutive layers is 25. The rock used as capping material has a carbonate nature able to create an alkaline environment, which facilitates the hydrolysis of chemical agents. Special additives are generally added to the rock in order to accelerate the hydrolysis process further.

Due to the pressure gradient between the wreck and the external part of the embankment, movements of the interstitial water could be generated leading to the spreading of chemical agents leaking from the ordnance. It is therefore

necessary to reduce the permeability of the embankment to at least 0,4 mm/h (a typical value for muddy-sandy sediments). This could be achieved through different methodologies:

- covering the external layer with flexible waterproof sacks filled with cement
- injection of cement into the embankment
- Use of a special, waterproof and erosion resistant sheet on the top of the embankment.

Capping of ordnance

Due to the high costs of these operations the use of the technique previously described it is not advisable. The areas affected in the Southern Adriatic Sea, are in fact numerous and of different dimensions, which would require a huge effort to remedy the situation. Moreover, the operations would cause significant modifications to the sea bed, as far as its nature and granulometry are concerned, leading to further modification of the benthic ecosystems involved. Strategic depressions on the seafloor could be created instead, where the ordnance detected in the vicinity could be dumped. The war material could be collected and transferred to the hollow using a R.O.V. and finally covered following the same procedure utilised for the capping of wrecks. The hollow could be created by suction devices connected to the R.O.V. (Fig. 7–6) or through a high pressure hydraulic pump able to remove the sediment.

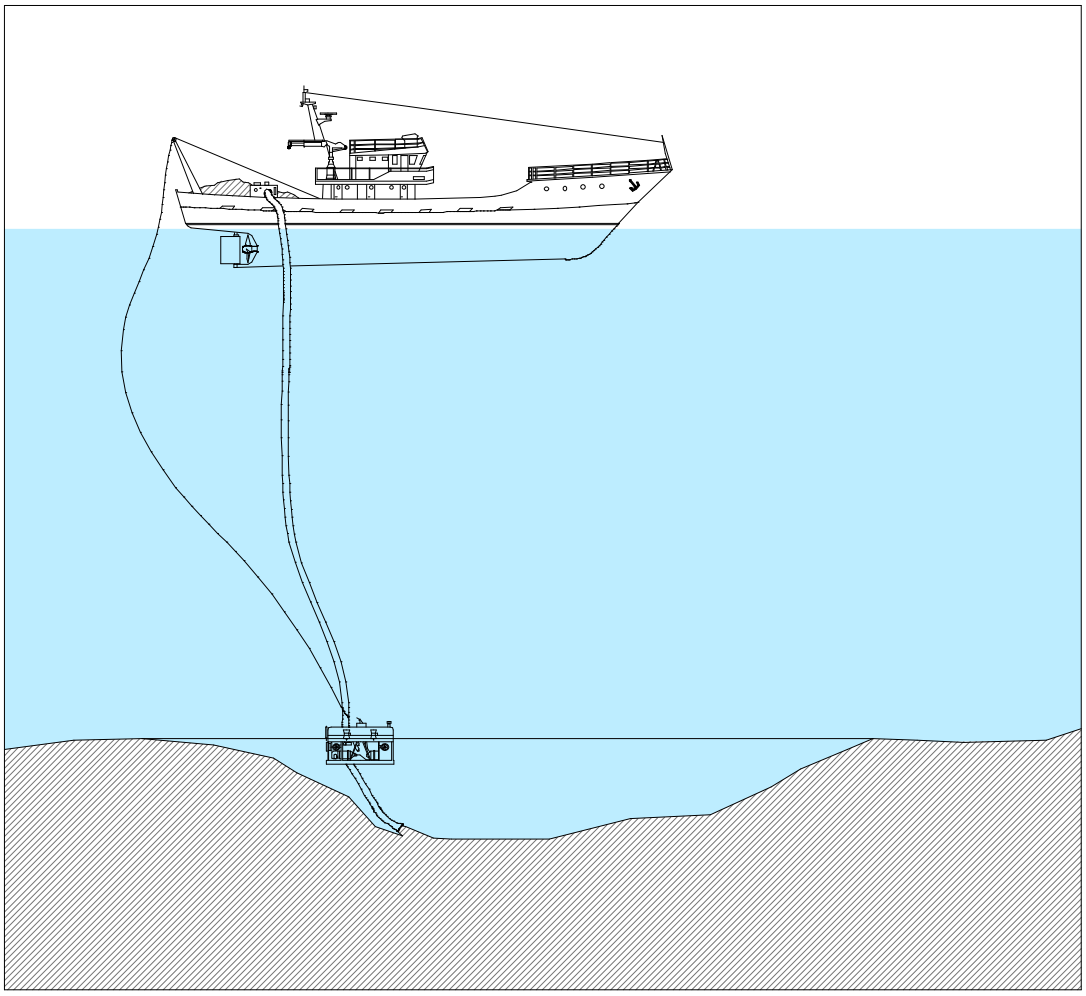


Fig 7–6 Creation of a hollow by a suction device mounted on a R.O.V.

If the CW dumping area is located at a depth greater than 100 metres the creation of a hollow is not possible for reasons of hydrostatic pressure. In this case the chemical weapons could be heaped together on the seafloor by a ROV and they could be capped in the same way as the wrecks.

It has to be noted that the displacement of aerial bombs in particular, which usually only have a shell of 1.5 – 2 mm in thickness, could lead to a partial leakage of their contents and to the consequent contamination of the equipment.

7.2.2 Recovery and disposal

A mechanical recovery operation of munitions using a remotely managed device was carried out in 2004 by Typhoon Contractors LTD in Israeli waters

(personnel communication Mr. Ayalon Roitemberg of Typhoon). This activity was necessary in order to clear the path for a future gas pipeline. The clearance process was carried out along a path of 18 Km using an underwater hydraulic, pneumatic jetty and a hydrostatically-operated stringer, particularly designed for this task, pulled by a barge and a tug boat (Fig. 7–7 and 7–8).

Fig. 7–7 Hydraulic pump (source: Typhoon Contractors LTD)

Fig. 7–8 Hydrostatically-operated stringer (source: Typhoon Contractors LTD)

The dredging operation created a channel 30 meters wide and 4 meters in depth. The recovery rate was verified by a ROV that shows a 100% efficiency.

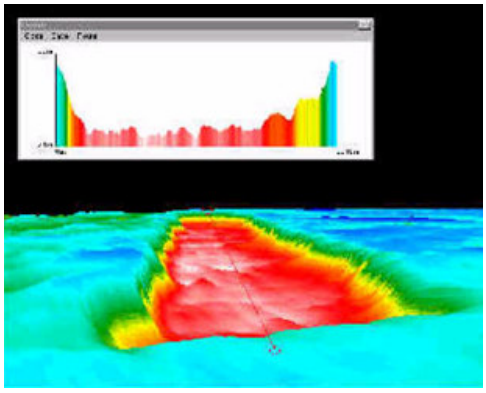


Fig. 7–9 The channel on the seabed produced by dredging operation and monitored by a multibeam (source: Typhoon Contractors LTD)

Fig. 7–10 Some old munitions recovered during the dredging operation (source: Typhoon Contractors LTD)

The limitation of this technology is that it works only at a depth of less than 100 metres, for reasons of hydrostatic pressure. The methodology could be usefully employed in shallow waters characterised by a soft sea bottom. During the decision making phase the following aspects should be taken into account: the possible acute release of chemical agents into the water due to the violent mobilisation of rusted chemical munitions, as well as benthic ecosystem alteration caused by the dredging operation.

The recovery of the dumped ordnance can also be accomplished through a specifically equipped R.O.V. able to transfer the material to a container which can then be recovered by the support vessel (Fig. 7–11 and 7–12).

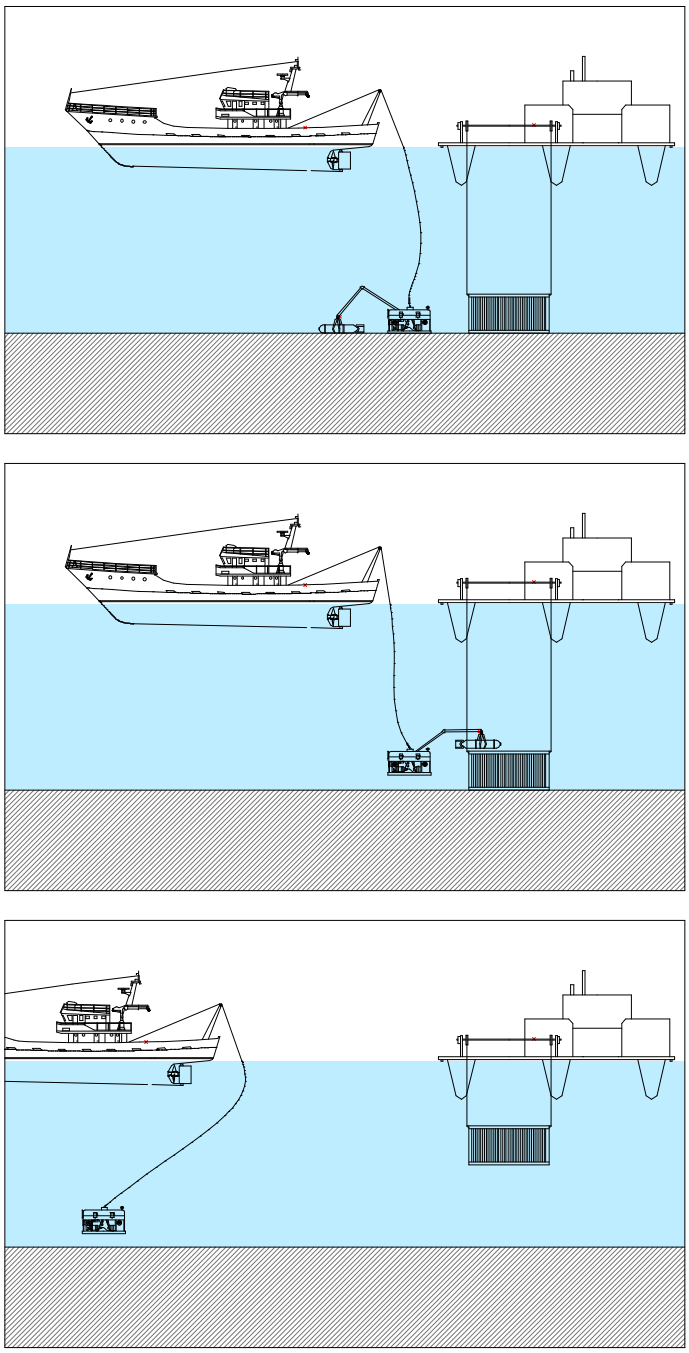


Fig 7–11 Recovery operation of CWs by a ROV

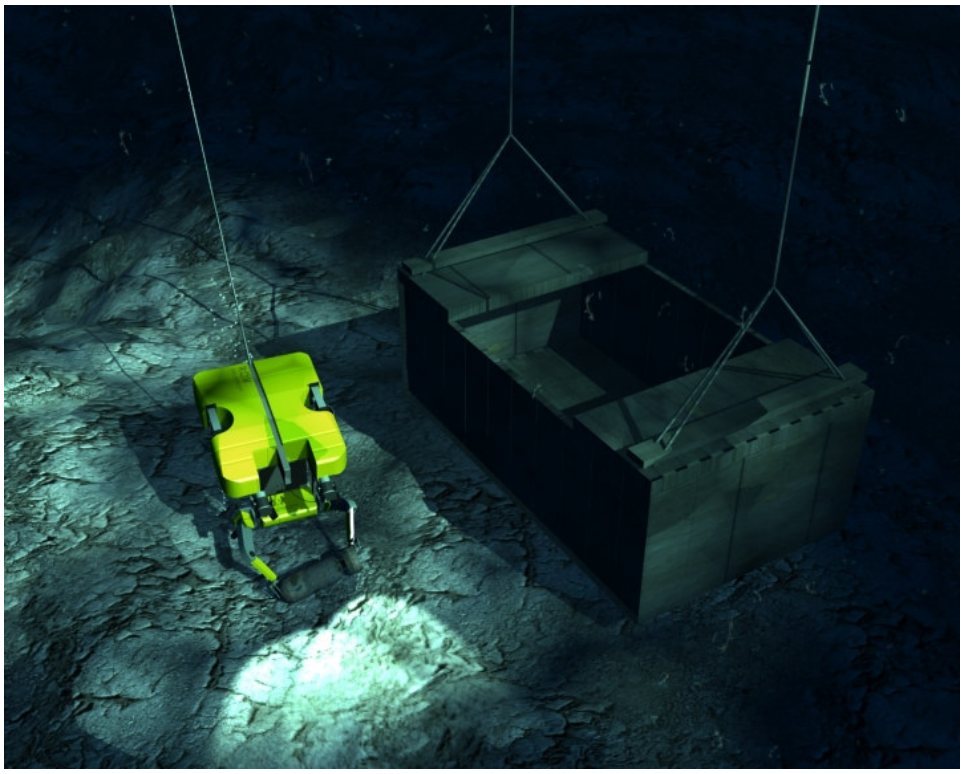


Fig 7–12 Details of the recovery operation

Thanks to the technological advances it is possible nowadays to recover around ten bombs per day. This means that nearly 6 years of work would be necessary to recover the supposed 20,000 ordnance dumped in the Southern Adriatic Sea. Moreover, both their displacement and the amount of pressure they would be exposed to once moved to the sea surface, would certainly increase the probability of shell breakage with consequent contamination of the equipment and creating a high risk situation for the operators.

The recovery of wrecks loaded with chemical weapons may also be technically feasible. It is necessary, however, to consider their weight, their state of conservation and the risks involved in moving the ordnance to the sea surface due to the sharp pressure variation. The difficulty of getting the weapons out of the holds needs to be considered as well.

7.2.2.1 DISPOSAL OF CHEMICAL WEAPONS

The recovered ordnance may either be disposed of on-site using a specific pontoon or transferred on land to a proper disposal site. We believe the first option is the most suitable, mainly for safety reasons regarding the civil population. The choice of the second option for cleaning up the CW dumping

areas in the Southern Adriatic Sea would mean the transportation of recovered, rusted munitions to the dismantling plant *Centro Tecnico Logistico Interforze NBC* (CETLI NBC) located at Civitavecchia (Rome). This is the only military institution authorised by the Chemical Weapons Convention (CWC) to deal with chemical weapon demilitarisation. The Centre is about 500 Km from Molfetta harbour, the nearest possible location where the unloading operation of recovered chemical weapons from ships could be carried out.

On-site disposal would involve the realisation of a pontoon where the following structures could be built: a weapon demilitarisation area, a field laboratory, a clean up area for materials and personnel and a remote control station for underwater instrumentation. The disposal area must be a closed environment with negative pressure inside to prevent accidental vapor release.

According to the CWC, chemical agents present in the Contracting Parties' territory need to be transformed in order to avoid any further use of these agents in chemical weapons. Three disposal methodologies which involve both physical and chemical treatments can be employed: explosion of munitions in controlled conditions, incineration, chemical degradation and transformation. Lately, the possibility of utilising bacterial degradation has also been explored. The choice of more than one possible methodology can also be considered.

Before proceeding with the demilitarisation of collected munitions, an analysis of their contents is often necessary in order to decide which procedure to adopt. A series of techniques exist, which are able to give information on chemical content without braking the munition shell (non-intrusive assessment technologies).

In the U.S. Army Non-Stockpile Chemical Material Program (NSCMP)[298] the US Army carried out a series of trials aimed to deal with and to solve all the problems related to the non-stockpile chemical munitions found in US territory. The portable isotopic neutron spectrometer (PINS), developed in the NSCMP project, can identify elements within closed munitions by detecting gamma rays, which are similar to x-rays, emitted by them if hit by a neutron source. All chemical compounds are composed by two or more elements. The presence and relative concentration of a specific chemical element can be determined by the characteristic gamma-ray peaks. Analysing the characteristic gamma-ray peaks potentially allows for the identification of compounds.

[298] For more details see http://www.cma.army.mil/nscmp.aspx

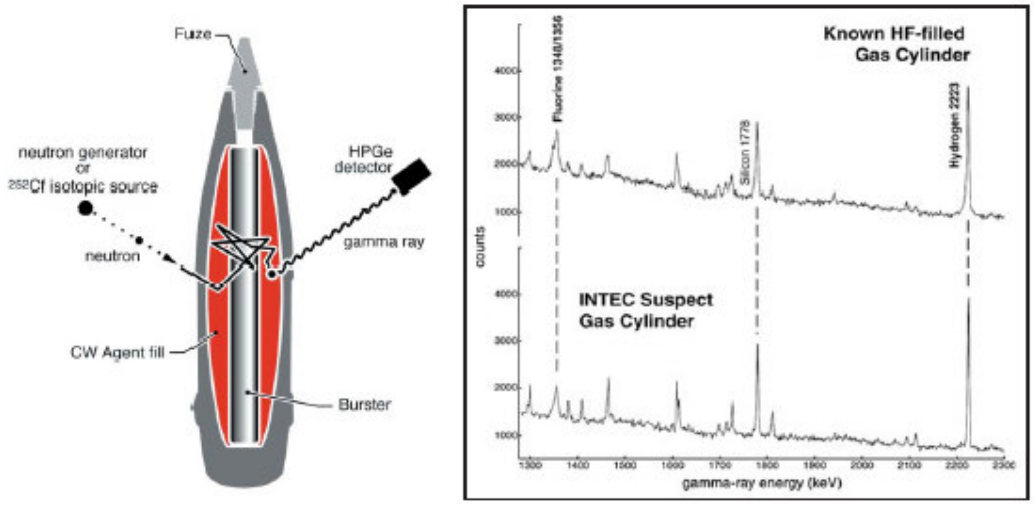


Fig 7–13 The portable isotopic neutron spectrometer (PINS) system.

(source: www.ortec-online.com/pdf/pins.pdf)

The PINS system uses three primary pieces of equipment to identify elements (Fig. 7–13). The first, a neutron source, is located near the item being analysed. Some of these neutrons penetrate the munitions shell and interact with the contents. The second piece of equipment is a gamma-ray detector. This piece of equipment monitors the energy and intensity of gamma-rays released by neutron interactions. In addition, a multi-channel analyser supplies power and receives electrical impulses from the gamma-ray detector. The voltage of each impulse is proportional to the gamma-ray energy. These impulses are amplified and sorted according to voltage into many channels.

The CETLI NBC, mentioned above, carry out preventive x-rays of all munitions recovered using radiographic technology. A digital image of the interior of the munition is obtained, which helps to identify any liquid contained within the war material and whether or not it is explosive. The physical status of munition content is obtained by tilting the bomb during the x-ray: if the compound is a liquid the surface remains horizontal (Fig. 7–14).

Fig 7–14 Radiography of a U.S. 155 – millimetre Howitzer Chemical Shell M105B1 filled with mustard gas (source: CETLI NBC).

A procedure to distinguish chemical weapons filled with a mixture of yperite and phenyldichloroarsine from those containing chloroacetophenone and chloropicrine (typical projectiles founded in Italian territory) is to x-ray the munitions at an environment temperature value between the melting temperatures of the two mixtures. 40 projectiles per hour can be x-rayed by the device.

The exploding of munitions in controlled conditions

A series of portable detonation chambers exist, which are capable of destroying chemical munitions on-site, by placing them into a pressure resistant cylinder and exploding them.

The Explosive Destruction System (EDS), developed in the NSCMP programme mentioned before, destroys chemical-filled, explosively configured munitions. It is a useful tool to destroy on-site munitions deemed unsafe to transport.

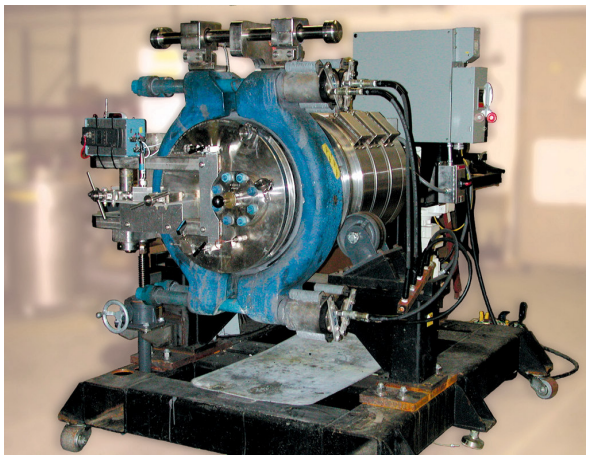
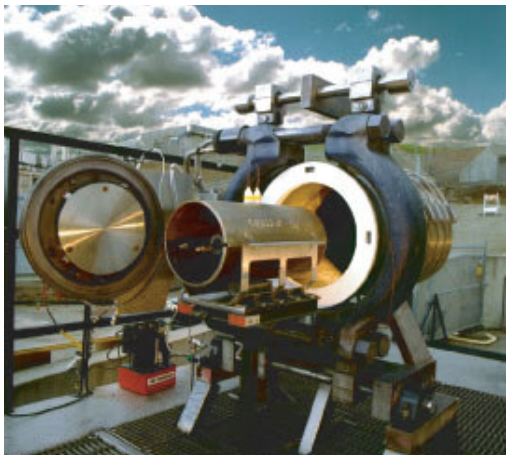



Fig 7–15 Explosive Destruction System (EDS)

(source: www.sandia.gov/news/resources/releases/2005/def-nonprolif-sec/bio-EDS.html)


Fig 7–16 On the left a mock 75mm munition with linear shaped charge in place; on the right the same post test.

(source: www.ca.sandia.gov/industry_partner/Demil/EDS_fact.html)

The system is able to contain the blast, the agent and the vapour of the treated munitions completely, in a 2-inch thick stainless steel vessel with a 7-inch thick door. The cylinder holds the munitions and contains the metal fragments during the detonation. Shaped charges for opening the munitions and destroying the explosives in the munitions are carefully placed before adding end caps and detonator wires (Fig. 7–16). After the detonation, operators add, by remote control, a chemical designed to neutralise the chemical agent in the munitions . EDS then slowly heats the liquid and begins a rotation phase that ensures complete neutralisation. Liquid waste drains into 55-gallon drums and the stainless steel chamber is rinsed with clean water. Finally, liquid and solid waste is packaged and shipped for disposal.

Very similar technology has been realised by CH2MHILL with the Controlled Detonation Chamber (CDC) capable of destroying both conventional and

chemical weapons (Fig. 7–17). Their massive structural parts made of high-strength steel are capable of absorbing and dissipating detonation shock waves, explosion heat and accelerated fragments. The CDC is also able to steadily release stabilised post-explosion gases.

Fig 7–17 The Controlled Detonation Chamber (source: CH2MHILL)

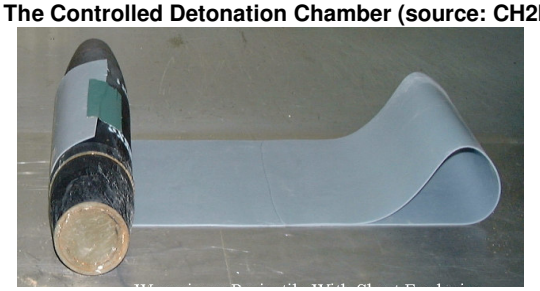


Fig. 7–18 Wrapping a projectile with sheet explosive (source: US Army Corp of Engineers)

Fig 7–19 An artillery bomb before and after the explosion in the CDC (source: CH2MHILL)

The CDC was used to destroy chemical weapons in a test conducted together with the US Army (Edgewood Chemical and Biological Center - ECBC) where a rate of about 4 munitions per hour was demonstrated (personnel communication

by Mr. Brint Bixler - CH2MHILL). Moreover, The CDC was also used in 2005 to destroy 2,500 old chemical weapons recovered in Belgium containing mainly diphenylchloroarsine (Clark I), yperite, phosgene and white phosphorus. One replacement of fragmentation protection was need after 2,000 detonations.

Incineration

All chemical agents are inflammable (with the exception of sarin which is not present in the Southern Adriatic) and their combustion, if properly controlled, can be complete. Until 2000 the American disposal plant for chemical weapons located in the Pacific, on Johnston Atoll (one-square mile island 800 miles southwest of Hawaii), used an incineration process to dispose of war materials. Using the Johnston Atoll Chemical Agent Disposal System (JACADS) the US Army destroyed over 400,000 obsolete chemical weapons collected from Okinawa and other U.S. military bases in the Pacific Basin and West Germany (Fig. 7–20).

Fig. 7–20 Chemical weapons stockpile on Johnston Atoll (source: US EPA http://www.epa.gov/region9/features/jacads)

The process involves a preliminary combustion stage carrried out at 500°C for 15 min., followed by a post-combustion stage at 1,200°C for 1 sec. Depending on the toxic agent involved, N, As, P, HCl and Cl_2, oxides can be produced following incineration. In order to avoid the production of dioxins, which are generally formed between 180 and 400°C, the cooling of the fumes is carried out very rapidly using water at room temperature. Any noxious emissions are avoided by the final absorption of the fumes through active carbon and the removal of hydrochloric acid and chloride with Na_2CO_3 solutions. In order to avoid any accidents this methodology, although very rapid, requires continuous monitoring and maintenance.

Fig. 7–21 Chemical weapons incineration plant on Johnston Atoll (source: Washington Group International http://www.wgint.com/docs/at/JACADS.pdf)

Chemical degradation

The methodology described below is utilised by the CETLI NBC. After the radiographic process described before, the chemical ordnance (drums and munitions) are cut and the contents collected in 3,000 litre containers.

The bomb shells are decontaminated by washing them alternately with HNO_3 solution and a highly basic solution, followed by a final wash with hot water to eliminate residual chemical agents.

The chemical agents (mainly yperite and yperite - phenyldichloroarsine mixture) collected in 3,000 litre containers are transformed into less harmful compounds by adding H_2O_2 at 50%, and are thus transformed into oxidised compounds like divinyl sulphone, divinyl sulphoxide, bis 2–chloro ethyl sulphone, bis 2-chloro ethyl sulphoxide, etc.. Milk of lime is added in order to increase the pH value, neutralising the compound.

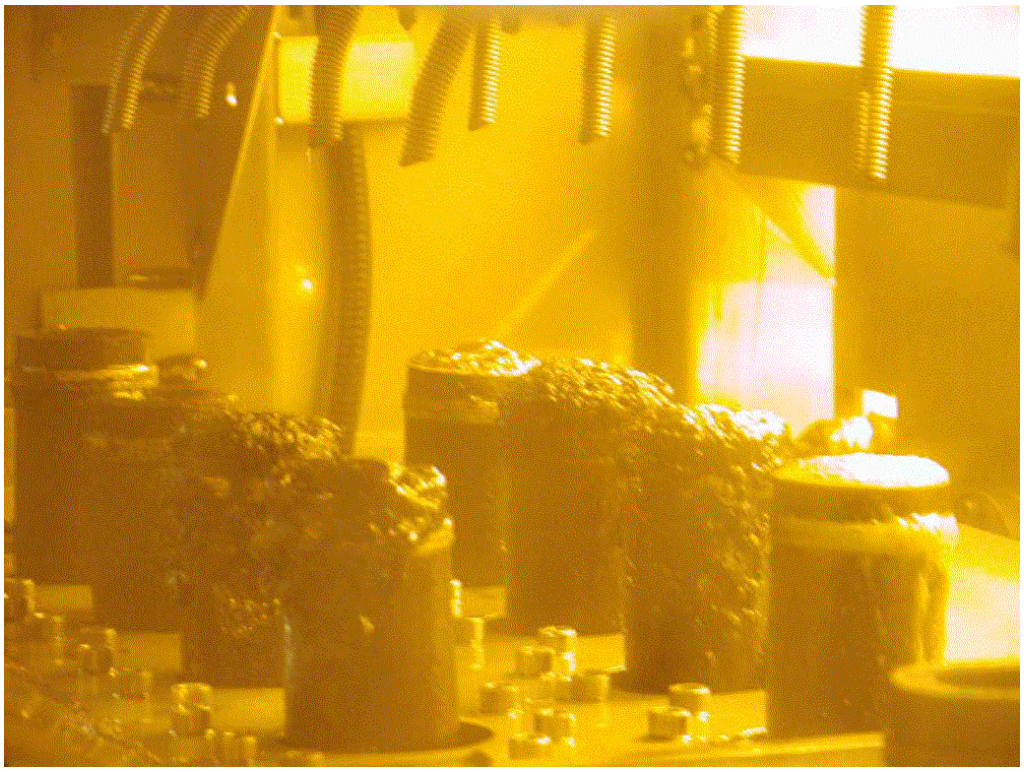


Fig. 7–22 Decontamination phase of chemical agents (source: CETLI NBC)

The products are finally mixed with cement, producing cylindrical blocks which are stored and monitored continuously in order to verify that there is no leakage of toxic molecules (Fig. 7–23).

Fig 7–23 Stockpile of cylindrical blocks of degraded chemical agents – cement mixture (source: CETLI NBC)

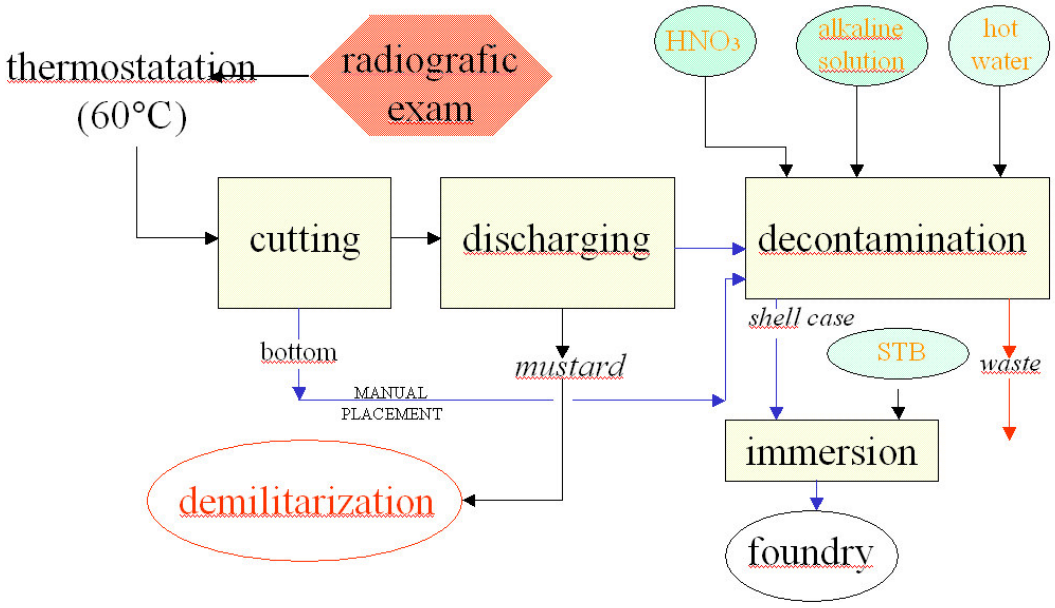


Fig. 7–24 Flow chart related to the demilitarisation process adopted by CETLI NBC (source: CETLI NBC)

8 Conclusions and recommendations

The results of the RED COD project validate the public and scientific interest on the matter and show the need for further studies and remediation activities.

The integrated ecotoxicological approach utilised in the study has shown to be effective in highlighting several concerns regarding particularly the consequences of CWAs leaking from rusted chemical weapons shells. Alterations at biochemical, cellular and tissue levels within the organisms sampled in the concerned CW dumping site, have been shown, particularly the clearest evidences have been observed in fish tissues for As tenors, dermal blisters and DNA damages.

The study on the effects of the release of TNT underlines as well a potential threat for the concerned benthic ecosystems. Laboratory results show significant differences between the values of stress indexes observed in samples collected within the study area versus the ones recorded in samples collected in the reference site. In particular, a significant increase of protein Hsp 70 in coelomocytes of *Paracentrotus lividus* and a reduction of hepatic P450 enzyme activities (in particular EROD activity) in *Conger conger* were observed.

The paucity of accounts related to ammunitions dumping operations and the practice adopted by fishermen to dislocate the ammunitions accidentally collected by their nets in areas where they don't operate make a very challenging task the mapping of the concerned sites on the seafloor. The methodology applied in this research in order to identify the main warfare dumping areas seems to have been adequate to achieve a better knowledge on both position and extension of these areas as well as on the quantities and typologies of the dumped ordnance.

A consistent part of the project has been dedicated to achieve information on the Best Available Technologies for cleaning up the CW dumping sites at sea. Although, with reference to the Baltic Sea and the North Sea conditions, the scientific and technical literature indicate that it is better to leave the ammunitions undisturbed on the seabed, the authors of the present report suggest that a pilot study aimed at experimenting the best available technologies for remediation activities, in order to assess costs and risks both for operators and the environment, could help in taking a pondered final decision whether to act against the pollutant represented by CWAs on the seafloor.

On the basis of the results achieved by the present study the following recommendations to decision makers are proposed:

- to develop guidelines for fishermen on how to deal with dumped chemical munitions when accidentally recovered on board and to elaborate instructions about reporting;
- to highlight the concerned sites and to interdict any sea-bottom related activity within;
- to consider the establishment of compensation for fishermen who hand over to the competent authorities the ordnance accidentally collected;
- to achieve a better knowledge on both position and extension of the main dumping areas through interviews with fishermen (providing them with a specific questionnaire like the one reported in annex) and consultation of military and civilian archives;
- to achieve a better knowledge on both quantities and typologies of the dumped ordnance by surveying the dumping sites by means of side scan sonar coupled with magnetometers and a remotely operated vehicle;
- to plan and to carry out monitoring campaigns within the dumping areas;
- to foresee a pilot remediation activity in order to test the best available technologies and to evaluate the cost-benefits ratio.

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Annex I

Map of Italian sea dumping areas

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Annex II

Map of Southern Adriatic sea dumping areas

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Annex III

Questionnaire used to collect information regarding events of accidental recovery of chemical weapons at sea from fishermen

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Questionnaire for the Southern Adriatic Sea[299]

Surname and Name _____

Marine membership _____

Fishing vessel _____

Fishing gear □ **Trawl net** □ **Long-line** □ **Permanent nets** □ **Other (specify)**

Address (Tel.; fax.; e-mail)_____

1 – Have you ever accidentally recovered dumped ordnance while fishing in the Southern Adriatic Sea?

□ Yes

□ No

2 – If yes, what is the frequency of these incidents?

□ From 0 to 1 time per year □ From 2 to 5 times per year □ From 5 to 10 times per year □ Other (specify)

3 – Was the accidentally recovered ordnance exploded or unexploded?

□ Exploded □ Unexploded □ Both

4 – What do the bombs look like?

□ They are always the same types □ They are often different

5 - What is their state of conservation?

□ Old and rusted □ In good conditions □ Old but intact □ Other (specify)

6 – Which are the areas where you accidentally recovered the bombs?

□ Always the same area □ Several areas □ It may happen anywhere □ Other (specify)

7 – Are there any areas that you avoid where you would be sure to recover a bomb?

□ Yes, more than one (specify) □ Yes, one □ No

_____

[299] **The given information will be used only in the context of the present research. Personal information will be kept reserved following the dispositions of italian law n.675/1996**

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8 – What do you do when you recover a bomb?

□ We dump it immediately | □ We place it out of the fishing vessel and we notify the event to the authorities | □ We place it out of the fish vessel and we dump it in a specific area | □ We place it out of the fish vessel and we dump it as soon as possible | □ We keep it on board and we dump it as soon as possible | □ Other

9 – Do you mark the coordinates of the recovery and dumping sites?

□ Yes, always □ No, never □ Sometimes □ No, because we always dump them in the same area

10– Have you ever suffered any health problems connected with the accidental recovery of bombs?

□ Yes, sometimes □ Yes, always □ No, never

11 – If yes, when did the first symptoms appear?

□ Immediately □ After some minutes □ After one hour □ After some hours

12 – If yes, because of ...

□...the explosion of the bomb recovered | □...leakage of a liquid product | □... leakage of a gas product | □... leakage of a solid product | □... fire from the shell | □..nets "contaminated" by a dangerous substance

13 – Describe the symptoms

14 - Describe the colour of substance contained in the bomb

15 - Describe the odour of substance contained in the bomb

16 – Can you describe one or more bombs?

□ Yes □ No

Description (also with a drawing)	Note (insert other information not included in the questionnaire)

16 – Do you recognise any ordnance reported on the following pages?

□ Yes □ No

17 – If yes, please indicate the corresponding letter

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Position of munitions recovery areas

Area I

Distance from the coast (e.g. 35 nautical miles from Molfetta and 15 from Manfredonia)

Coordinates and depth of point where the nets are hauled down and hauled in

Hauling down point: Lat. (φ) Long. (λ) Depth (meter)

Hauling in point: Lat. (φ) Long. (λ) Depth (meter)

Extension (ex. Width 3 nautical miles)

Coordinates and depth of centre areas

Lat. (φ) Long. (λ) Depth (meter)

Coordinates and depth of vertexes of area

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Area II

Distance from the coast (e.g. 35 nautical miles from Molfetta and 15 from Manfredonia)

Coordinates and depth of point where the nets are hauled down and hauled in

Hauling down point: Lat. (φ) Long. (λ) Depth (meter)

Hauling in point: Lat. (φ) Long. (λ) Depth (meter)

Extension (ex. Width 3 nautical miles)

Coordinates and depth of centre areas

Lat. (φ) Long. (λ) Depth (meter)

Coordinates and depth of vertexes of area

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

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Area III

Distance from the coast (e.g. 35 nautical miles from Molfetta and 15 from Manfredonia)

Coordinates and depth of point where the nets are hauled down and hauled in

Hauling down point: Lat. (φ) Long. (λ) Depth (meter)

Hauling in point: Lat. (φ) Long. (λ) Depth (meter)

Extension (ex. Width 3 nautical miles)

Coordinates and depth of centre areas

Lat. (φ) Long. (λ) Depth (meter)

Coordinates and depth of vertexes of area

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Area IV

Distance from the coast (e.g. 35 nautical miles from Molfetta and 15 from Manfredonia)

Coordinates and depth of point where the nets are hauled down and hauled in

Hauling down point: Lat. (φ) Long. (λ) Depth (meter)

Hauling in point: Lat. (φ) Long. (λ) Depth (meter)

Extension (ex. Width 3 nautical miles)

Coordinates and depth of centre areas

Lat. (φ) Long. (λ) Depth (meter)

Coordinates and depth of vertexes of area

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

Lat. (φ) Long. (λ) Depth (meter)

***Get information about other areas if necessary**

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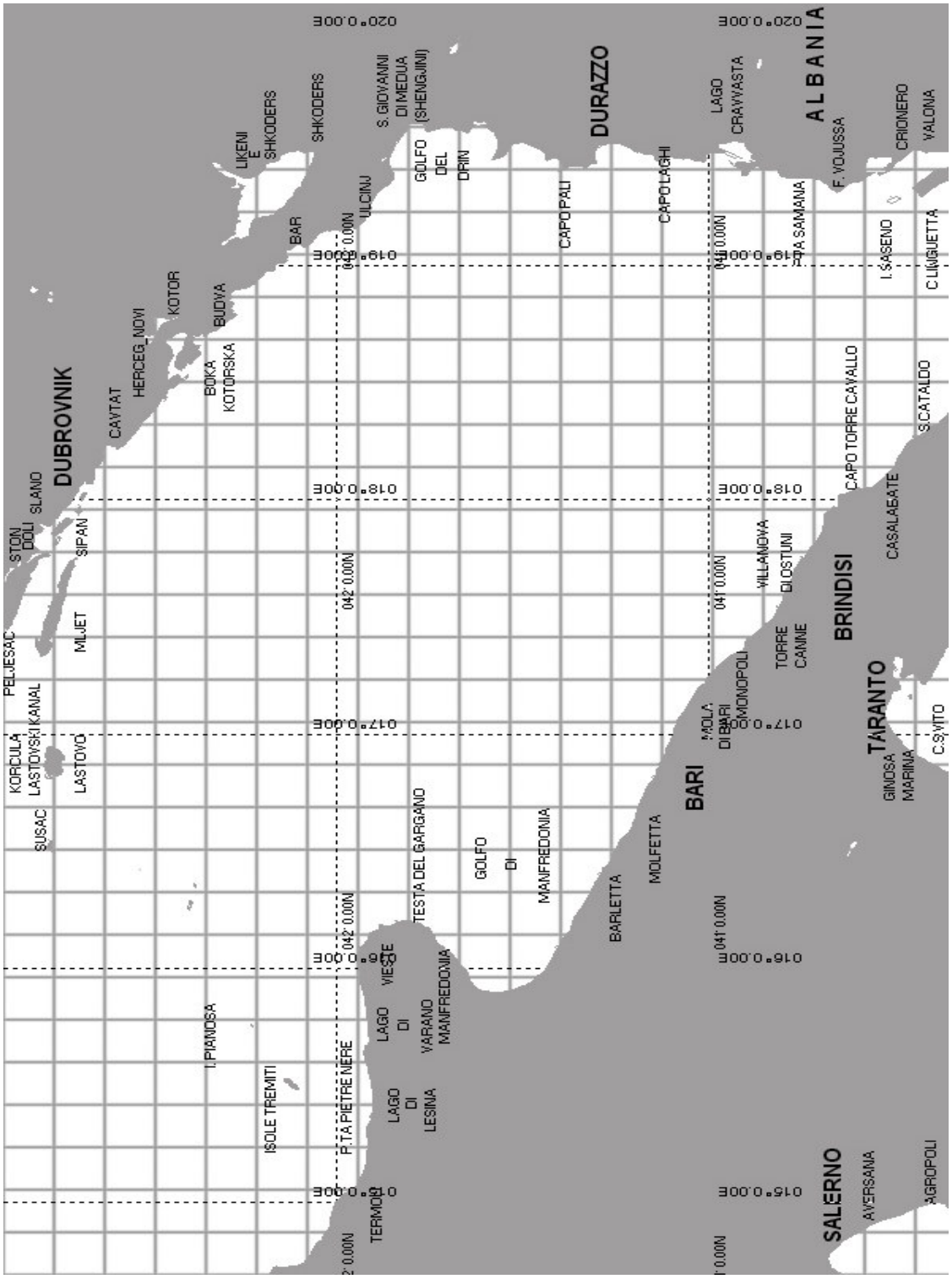
Blacken the squares on the map where you believe the main munitions dumping areas are positioned (the length of the side square is about 8 nautical miles)



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Annex IV

Main chemical weapons dumped in the Southern Adriatic Sea

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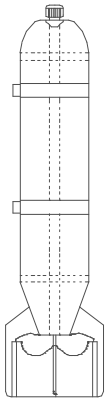

Description	Aerial bomb M47 A1 model. The bombshell consists of a steel sheet reinforced by two metal rings. Two hooks can be observed, which are used to handle the bomb.
Chemical Warfare agents	Yperite or White Phosphorus (inflammable)
Length (cm)	116
Diameter (cm)	21
Weight (kg)	75

Description	Mortar grenade with a vane characterised by six fins. If the device is in a secure position, a screwed on protects the spool.
Chemical Warfare agents	Yperite or Chloropicrine or Phosgene
Length (cm)	52
Diameter (cm)	11
Weight (kg)	12

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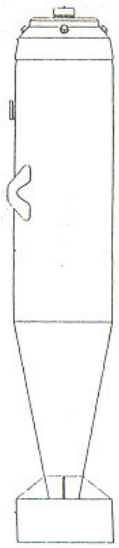

Description	Aerial bomb made with an iron cylindrical body, a rear conical side where the vane is mounted and a cylinder with four fins used to regulate the direction
Chemical Warfare agents	Yperite or White Phosphorus (inflammable)
Length (cm)	80
Diameter (cm)	12
Weight (kg)	unknown

Description	Aerial bomb able to disperse its contents when activated in a spray form.
Chemical Warfare agents	Yperite or Chloropicrine or Phosgene
Length (cm)	177
Diameter (cm)	33
Weight (kg)	unknown

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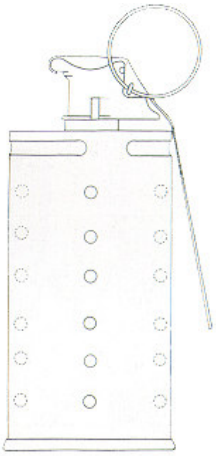
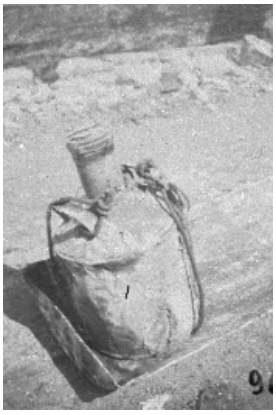
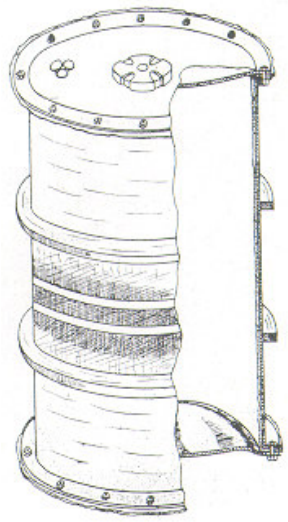
Description	Artillery bomb
Chemical Warfare agent	Yperite
Length (cm)	60
Diameter (cm)	75
Weight (kg)	unknown



Description	Cylindrical hand grenade
Chemical Warfare agents	Hydrogen cyanyde
Length (cm)	14
Diameter (cm)	6
Weight (kg)	unknown



Description	Drum like a demijohn. The bomb has a a cylinder provided with handles used for transporting purposes, a charging neck and a stopper to eliminate excess vapours.
Chemical Warfare agents	Yperite
Length (cm)	64
Diameter (cm)	30
Weight (kg)	45



Description	Similar to a fuel drum, the shell is made of an iron sheet covered by a lead layer. Two metallic rings are present on the central part of the body.
Chemical Warfare agents	Yperite or Diphosgene
Length (cm)	74
Diameter (cm)	52
Weight (kg)	about 400

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Description	Mortar bomb similar to a lamp. The body is divided into two parts: the front part may or may not contain a toxic liquid; the rear is charged with explosive or chemical agent.
Chemical Warfare agents	Phenol and cyanide compounds not specified
Length (cm)	38
Diameter (cm)	9
Weight (kg)	about 3

Description	Smoking drum
Chemical Warfare agents	Chlorosolfonic acid
Length (cm)	unknown
Diameter (cm)	unknown
Weight (kg)	95

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Annex V

Chemical warfare agents among the ones filling the ordnance dumped in Southern Adriatic Sea

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Annex VI

Flow-chart of CWAs behaviour in marine environment on the base of their chemical-physical characteristics[300]

[300] Modified from Helsinki Commission (HELCOM), 2002. HELCOM Manual on Co-operation in Response to Marine Pollution within the framework of the Convention on the Protection in the Marine Environment of the Baltic Sea Area (Helsinki Convention).vol. 2**.** www.helcom.fi

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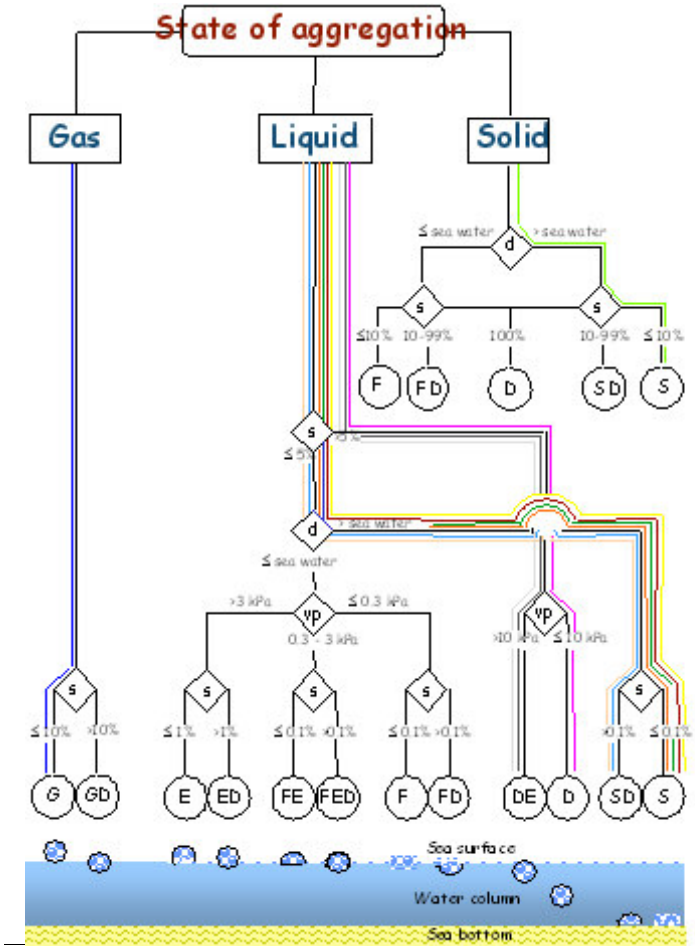

	Group	Properties
Evaporate Immediately (gases)	G	Evaporate immediately
	GD	Evaporate immediately Dissolve
Evaporate rapidly	E	Evaporate
	ED	Evaporate Dissolve
Float	FE	Float Evaporate
	FED	Float Evaporate Dissolve
	F	Float
	FD	Float Dissolve
Dissolve	DE	Dissolve rapidly Evaporate
	D	Dissolve rapidly
Sink	SD	Sink Dissolve
	S	Sink

s = solubility

d = density

vp = Vapour pressure

Yperite (H;HD)

Nitrogen mustard (HN3)

Nitrogen mustard (HN1)

Lewisite 1

Phosgen

Diphosgen

Cyanide acid

Cyanide chlorathe

Chlorosolphonic acid

Chloropicrine

Adamsite

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Annex VII

Information form for each specimen collected

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RED COD PROJECT

Southern Adriatic Sea Campaign 2004

Card n° **1**	Specimen cod. **M0704H**	Compiler **Marzia Umani**
Species ***H. dactylopterus***	Date **17/07/04**	Time of tissues collec. **12:30**
Total length. (cm) **32**	Weight (g) **1500**	Sex _____

- - - - - - - - - - - - - - - - - - -

Long line characteristics

Positioning date **17/07/04** Positioning time **09:00**

Recovery date **17/07/04** Recovery time **12:30 – 13:30**

Depth (m) **230** n° fishing hooks **500** Length (m) **1.200**

Initial point (co-ord.) **ϕ 41°46'.250N** Final point (co-ord.) **ϕ 41°46'.270N**
λ 016°52'.740E **λ 016°54'.690E**

- - - - - - - - - - - - - - - - - -

Sample collection

⊗ Otholits n° **2** ◊ Vertebras **No**

⊗ Liver n° samples stress indexes **1** n° samples CWAs **1** n° samples hystop. **1** n° samples DNA **1**

⊗ Gills n° samples stress indexes **1** n° samples CWAs **1** n° samples hystop. **1** n° samples DNA **1** n° samples metals **1**

⊗ Muscle n° samples stress indexes **1** n° samples CWAs **1** n° samples metals **1** n° samples DNA **1**

⊗ Blood n° samples micronuclei **5** n° samples hem.val: Plasma **1** Serum **1** n° samples CWAs **1**

◊ Gall-bladder n° samples CWAs **No** ⊗ Brain n° samples stress indexes **1**

⊗ Skin n° samples hystop. **1** ⊗ Rene n° samples hystop. **1** n° samples DNA **1**

⊗ Spleen n° samples hystop. **1**

⊗ Gonads n° samples sex determ. **1** n° samples DNA **1** ⊗ Gut n° samples DNA **1**

Total samples **30**

- - - - - - - - - - - - - - - - -

Note:

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Annex VIII

Age determination of *Conger conger* (L. 1758) based on otoliths analysis

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AGE DETERMINATION OF CONGER CONGER (L. 1758) BASED ON OTOLITHS ANALYSIS OF POST METAMORPHIC SPECIMENS

Introduction

Otoliths are bony structures located within the fish's skull whose dimensions are proportional to fish growth[301]. These are commonly used to determine the age of fish at macrostructural level, by counting the annual bands, hyaline and opaque, which are developed on the otoliths during the winter (slow fish growth), and the summer (fast fish growth) period respectively. A year of life is represented by the sum of a hyaline ring and an opaque ring [302].

Materials and methods

Otoliths were removed from the head of the fish on board, using a knife and a pair of forceps [303]. Both the sagittas were collected from the lower part of the saccular vestibule.

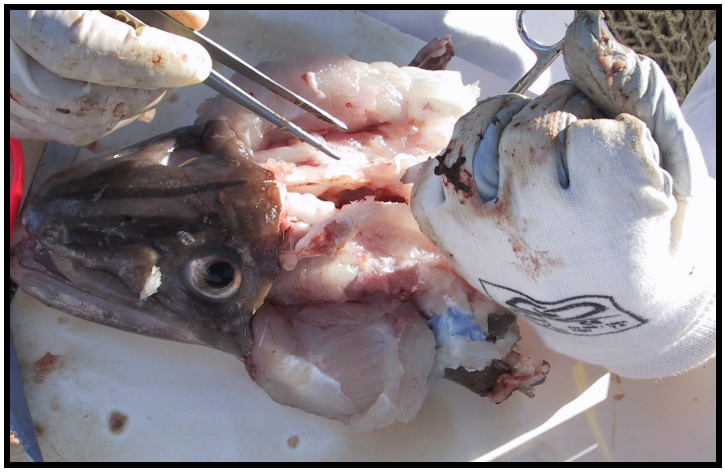

Fig. A: Otoliths removal from *Conger conger*

The otoliths were cleaned with water and stored dry in eppendorf.

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[301] Campana, S.E., Neilson, J.D. ,1985. *Microstructure of fish otoliths.* Can. J. Fish. Aquat. Sci.,**42**: 1014-1032.

[302] Giannetti G. and Donato F., 2003. *Fish Age Determination by Otolith Reading.* FAO-Adriamed Training Course GCP/RER/010/ITA/OP-08 Termoli (Italy)

[303] Secor D.H., Dean J.M. and Laban E.H., 1991. *Otolith Removal and Preparation for Microstructural Examination: a Users Manual.* http://cbl.umces.edu/~secor/otolith-manual.html

In laboratory, otoliths were weighted, measured and observed in a Petri disk with alcohol 70% under stereomicroscopy along the sagittal plane.

All the right otoliths were embedded in epoxy resin, left to dry for 12h and finally cut with micrometer, in order to obtain a section 0,7mm thick transversely through the core.

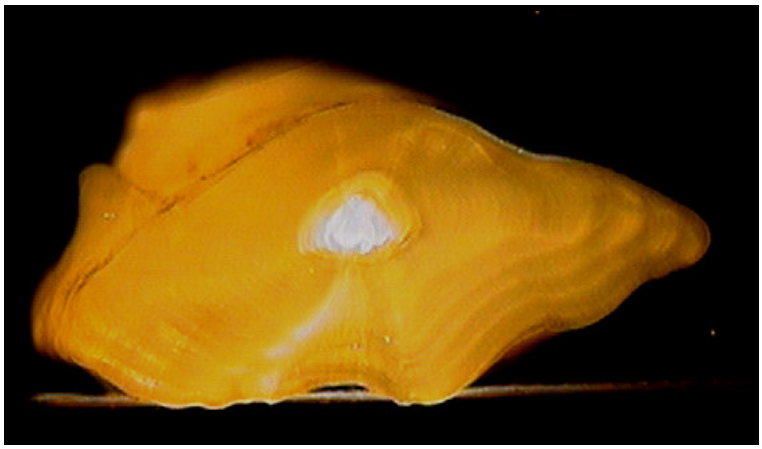

Fig. B: Sagitta of five years old *Conger conger* embedded in epoxy resin

All sections were polished and read at stereomicroscopy. A part of them were coloured with neutral-red in order to evidence the hyaline rings.

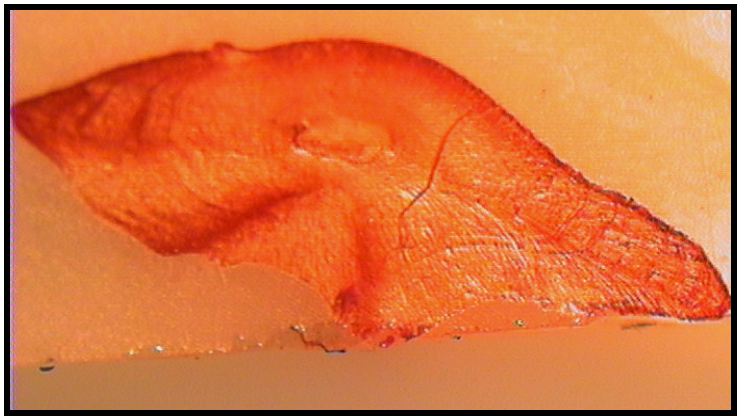

Fig. C: Sagitta of five years old *Conger conger* coloured by neutral-red

Age determination was carried out by observing the banding patterns using a binocular stereomicroscopy at 25X under reflected light.

Results

The range in length and weight of fishes was 66÷145 cm and 718÷7621g respectively. Age determination from sagittal otoliths ranged from 3 to 9 years, postmetamorphosis.

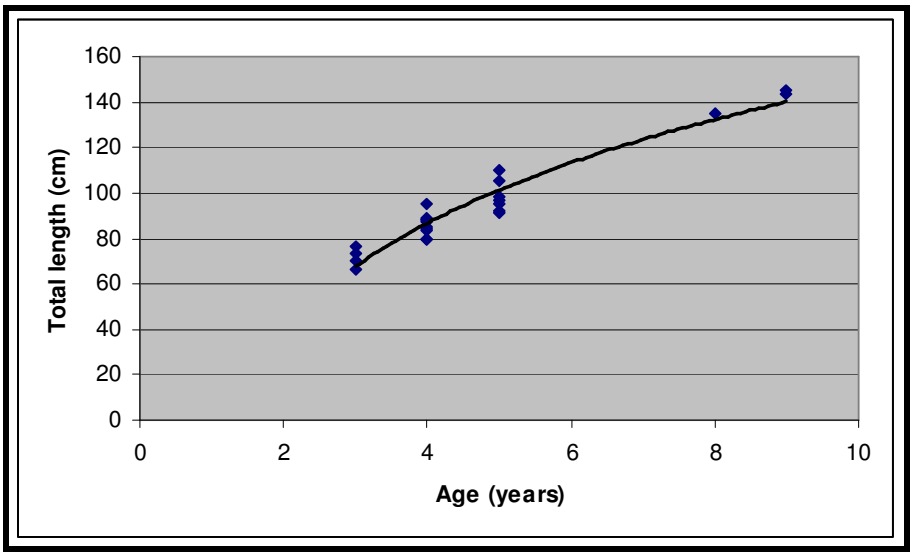

Fig. D: Growth curves fitted to the length-age data

Sections were clearer and thus easier to read than the entire otolith, while there were not many differences between coloured and uncoloured sections.

No significant differences in length or age were found between specimens collected within the two sites.

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Appendix

"Explanatory manual of the precautionary measures to adopt in the case of accidental recovery of war surplus in trawling nets"