Acute sublethal effects of 2,4,6-trinitrotoluene (TNT) on the European eel *Anguilla anguilla* (Linnaeus, 1758)

C. Della Torre[1], I. Corsi[1], C. Sensini[1], A. Arukwe[2] & S. Focardi[1]
[1]Department of Environmental Science,
"G. Sarfatti", Siena University, Siena, Italy
[2]Department of Biology,
Norwegian University of Science and Technology (NTNU),
Trondheim, Norway

Abstract

The aim of this study is to investigate the effects of the explosive 2,4,6-trinitrotoluene (TNT) on liver phase I and II biotransformation enzymes at gene (CYP1A1, GST and UDPGT) and catalytic levels by investigating EROD, GST and UDPGT activities, on crucial brain steroidogenic proteins (StAR and P450scc genes expression) and on gills histology in a model fish species such as the European eel (*Anguilla anguilla*, Linnaeus, 1758). Eels were exposed *in vivo* for 6 and 24 hours to 0.5mg/L, 1mg/L and 2.5mg/L nominal concentration of TNT by using 0.1‰ of DMSO as a carrier. The TNT produced a significant inhibition of EROD activity and an increase of UDPG T and GST genes expression and activities compared to controls. A decrease of StAR and P450scc genes was also observed in TNT exposed eels. Finally concerning gills, branchial lifting was evident at the lowest TNT concentration (0.5 mg/L) while lamellar aneurisms, vascular congestion, hypertrophy and hyperplasia of the epithelium were evident at 2.5 mg/L. Our results highlighted the concern related to the release of TNT on the seabed: the inhibition of EROD activity may result in an increased susceptibility of the organism to P450 inducers such as dioxins and PAH. TNT also seems to affect fish neurosteroidogenesis by downregulating key enzymes (StAR and P450scc genes). Gills seem to be a target organ for TNT in fish. The present research provided relevant information on TNT metabolism/toxicity and indicated sensitive targets of TNT toxicity in fish species.
Keywords: 2,4,6-trinitrotoluene, European eel, EROD, GST, UDPGT, neurosteroidogenesis, StAR, P450scc, gill histology.

1   Introduction

2,4,6-trinitrotoluene (TNT) is the one of the most common nitroaromatic explosives used in conventional bombs, and its presence in the marine environment is associated with military activities, ammunition manufacturing and extensive dumping of unexploded ordnance at sea. The acute and chronic toxicity of TNT reported for several aquatic species (Talmage *et al* [1]), highlight the concern related to the presence of the compound in the marine environment. In this view there is an urgent need to expand the knowledge on the metabolic fate and ecological impact of TNT on marine species focusing on metabolic pathways and targets of toxicity of this compound. In fact until now few investigations have focused on the molecular and biochemical pathways that determine TNT toxicity in aquatic species (Ek *et al* [2,3], Johnson *et al* [4], Sims and Steevens [5]). The aim of the present study is to investigate the acute effects of TNT on three different potential targets: phase I and II drug metabolizing enzymes cytochrome P4501A1 (CYP1A1), UDP-glucuronosyltransferase (UDPGT) and glutathione-S-transferase (GST); on brain neurosteroidogenesis key proteins such as steroidogenic acute regulatory (StAR) protein and CYP450 side-chain cleavage (P450scc) and on gill structure, using the European eel *Anguilla anguilla* (Linnaeus, 1578) as model fish species. The goal is to provide relevant information on noxious effects that could occur after an acute exposure to this compound in the marine environment.

2   Methods

2.1 *In vivo* exposure

Juveniles of European eels were exposed to waterborne TNT dissolved in DMSO (0.1‰) at concentrations of 0.5, 1 and 2.5 mg/L; in addition, one group (as control) exposed to 0.1‰ DMSO and a blank in marine water were maintained during the entire experiment. The TNT concentrations chosen were below or in the range of the 96-h LD50 calculated for fish (0.8–3.7 mg/L) [1]. Once absorbed by aquatic organisms TNT is readily metabolized with a half life measured in laboratory exposed fish of 0.05 h and a very fast elimination (Ownby *et al* [6]). In this view the acute exposure via water was selected in order to evaluate noxious effects of the compound in the most realistic exposure condition occurring in the marine environment in dumping areas. Eels were sacrificed after 6 and 24 hours. Livers and brains were excised and stored at -80°C. Gills second right branchial arches were dissected out and fixed in Bouin solution for histological analysis.

2.2 Molecular analysis

CYP1A1, GST and UDPGT transcripts in liver and StAR and P450scc in brain were investigated by quantitative real-time PCR using primers designed from conserved regions of the selected genes using PCR designer software PRIMER3. Primer pair sequences and their amplicon size are shown in Table 1.

Table 1: Primer sequences and amplicon size.

| Target gene | Primer sequence | | Amplicon size | Annealing temperature |
	Forward	Reverse	nucleotides	°C
CYP1A1	GGAGGGTGAGTACCTGGTGA	GAGTTCCTGGTCATCGTGGT	147	60
UDPGT	ATAAGGACCGTCCCATCGAG	ATCCAGTTGAGGAAGG	112	55
GST Pi	ATCACCTACTTTGCGGTTCG	GGCCCAGATGTCTGAGGATA	208	60
StAR	TCAGCATCCTCAGTGACCAG	CAGCTCCCCATACAGGTTGT	151	60
P450scc	ACAGGAGTCAGGTGGTGAGG	TGTCTGGCCAGCTCATACAG	167	60

2.3 Biochemical analysis

EROD, GST and UDPGT activities were measured in liver microsomal fraction of eels by biochemical assay following methods of Burke and Mayers [7], Habig *et al* [8] and Collier *et al* [9], respectively. Total proteins content was measured by the method of Bradford [10].

2.4 Histopathology

For histological studies the second right branchial arch was dissected out. Gills were fixed in Bouin solution, prepared in a routine manner and embedded in Technovit 7100 resin; sections 3-5 μm thick were cut with an LKB Ultratome III and stained with Mayer's haemallum and eosin (Bio-Optica) (Culling *et al* [11]). Sections were examined by Olympus BX 51 light microscope and images were taken with an Olympus digital camera (DP 50).

2.5 Statistical analysis

Comparison of TNT treatments and time of exposure (6 h and 24 h) was made by the non-parametric Mann-Whitney-Wilcoxon rank sum test. $P = 0.05$ was considered as maximum significant value. Statistical analyses were performed with Statistica 5.1 (StatSoft, USA).

3 Results

3.1 Phase I and II genes expression and enzymes activities

No modulation of CYP1A1 gene expression was observed within 24h of exposure (Fig. 1). On the opposite, EROD showed a significant decrease at the lowest TNT concentration (0.5 mg/L) with respect to controls and a further decrease was observed at higher concentrations (Tab. 2) stronger at 24 h than 6 h. TNT also dose-dependently increase the expression of UDPGT transcript at 6h, with significantly higher values than controls at 1 and 2.5 mg/L. UDPGT expression decreased slightly at 24h (Fig. 2). At enzyme level, no effects were observed at 6h, while at 24h an increase was observed at the highest TNT

concentration (Tab. 2). GST gene expression showed a dose-dependent increase only at 24 h (Fig. 2) while GST activity significantly increases only at the highest TNT concentration (Tab. 2).

Table 2:   EROD, UDPGT and GST activities as mean ± standard error; N=4; *significant difference with respect to DMSO group (p<0.05).

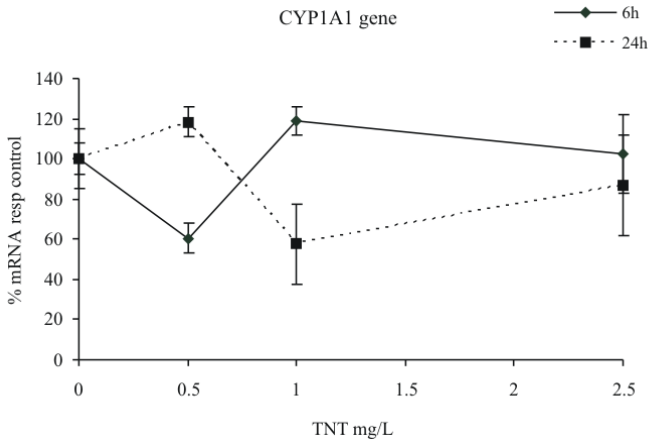|	Time	DMSO	0.5 mg/L	1 mg/L	2.5 mg/L
EROD	6h	110.17 ± 5.98	78.24 ± 5.27*	63.52 ± 2.59*	66.97 ± 5.93*
pmol/min/mg prot	24h	97.27 ± 10.17	47.04 ± 7.77*	44.33 ± 7.38*	40.21 ± 4.78*
UDPGT	6h	35.46±1.31	34.53±0.54	35.94±2.26	36.87±2.06
nmol/min/mg prot	24h	29.83±0.75	43.69±3.08	38.34±1.07	40.37±2.16*
GST	6h	443.04 ±65.24	561.78±40.40	541.49±73.57	1043.73±94.29*
nmol/min/mg prot	24h	477.56 ±20.85	319.35±56.44	521.97±58.72	1197.41±61.77*

Figure 1:   CYP1A1 gene expression.

3.2 Neurosteroidogenesis key proteins

StAR and P450scc displayed a similar response pattern to TNT exposure (Fig. 3). Gene expression was significantly decreased in a concentration-specific manner at 6 h with 0.5 and 1 mg/L, while at 2.5 mg/L, the mRNA expression levels were restored to controls. No further modulation was evident after 24 h of exposure for both genes.
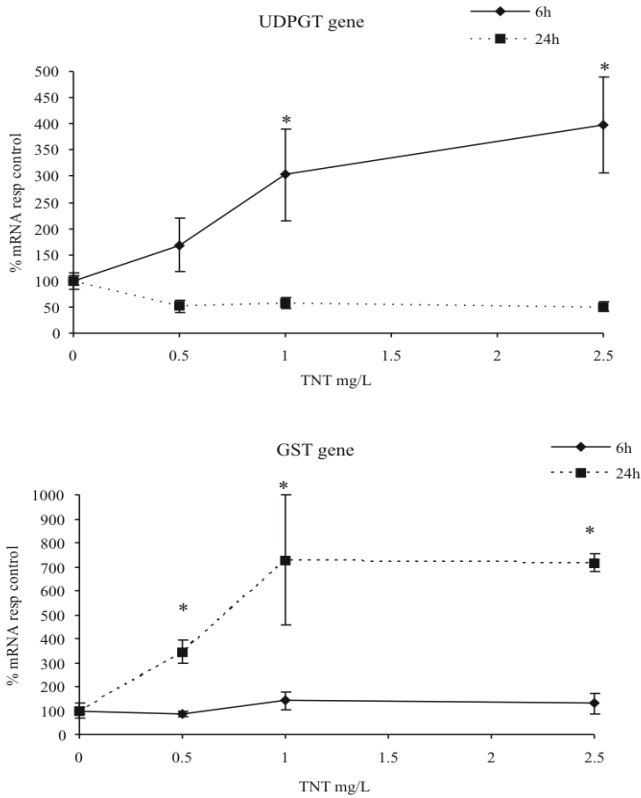
Figure 2:   UDPGT and GST gene expression. *Significant difference with respect to DMSO group ($p<0.05$).

3.3 Gill histopatology

Gills of sea-water control (a) and of DMSO exposed eels (b) showed normal histology of the secondary lamellae. Fish exposed to TNT showed a series of histological alterations. After 6 h, pathological effects in the secondary lamellae were evident: at 0.5 mg/L and 1 mg/L TNT gills showed oedema of some secondary lamellae. Both at 1 and 2.5 mg/L this change led to epithelial lifting and only at 2.5 mg/L a detachment from the endothelium and rupture of the branchial membrane and vascular congestion were observed (Fig. 4d, 4e). After 24 h, at 0.5 mg/L a greater damage with increased oedema in secondary lamellae, extensive epithelial detachment and vascular congestion were observed (Fig. 4c, f). At 2.5 mg/L, distortion and protrusion of lamellae leading to epithelial hyperplasia, fusion of adjacent lamellae and obliteration of interlamellar spaces by means of tight junctions were observed (Fig. 4g). Chloride cells proliferated along secondary lamellae and mucus hypersecretion was evident at 2.5 mg/L with lamellar blood sinus constriction and hyperplasia (Fig. 4g). At this
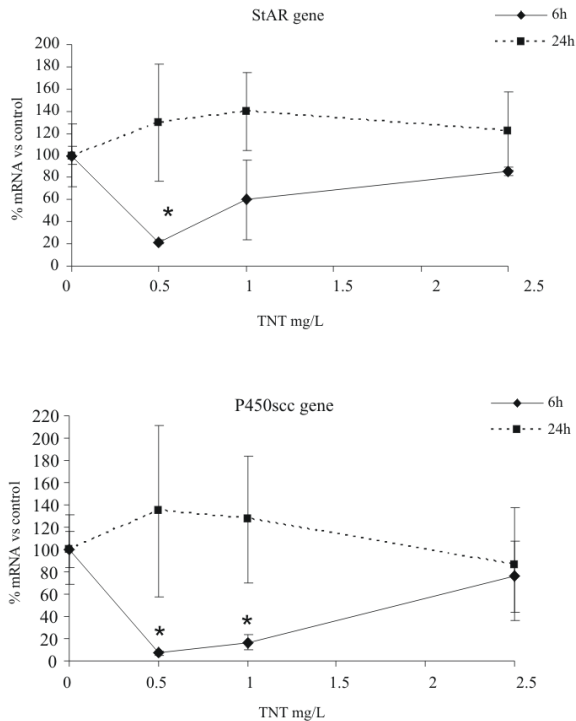
Figure 3:   StAR and P450scc expression. *Significant difference with respect to DMSO group (p<0.05).

concentration the permeability of capillaries walls increases, producing an exudation of fluid that leads a congestion of blood cells in these vessels (vascular stasis). Dilation of lamellar capillaries and pooling of blood were evident at both time of exposure (Fig. 4e, g).

4   Discussion and conclusions

Results showing inhibition of EROD by TNT suggest that it could competitively inhibit oxidation of other substrates by CYP1A such as classical inducers i.e. dioxins and PAHs. Since TNT is metabolized within few hours of exposure [6], aminated metabolite could, reasonably, be involved in generating the inhibitory effect. The dose-dependent increase of phase II genes encoding UDPGT and GST and related catalytic activities are consistent with the involvement of phase II enzymes in TNT metabolism as already documented in other vertebrate species (Reddy *et al* [12]). More specifically the study of Ek *et al* [3] indicates TNT exists in fish bile as glucuronide conjugates. In laboratory mammals a major portion of TNT metabolites is excreted as glucuronide conjugates [1]. The time-response differences observed in the present study for UDPGT and GST genes and enzyme activities could be due to specific conjugation with
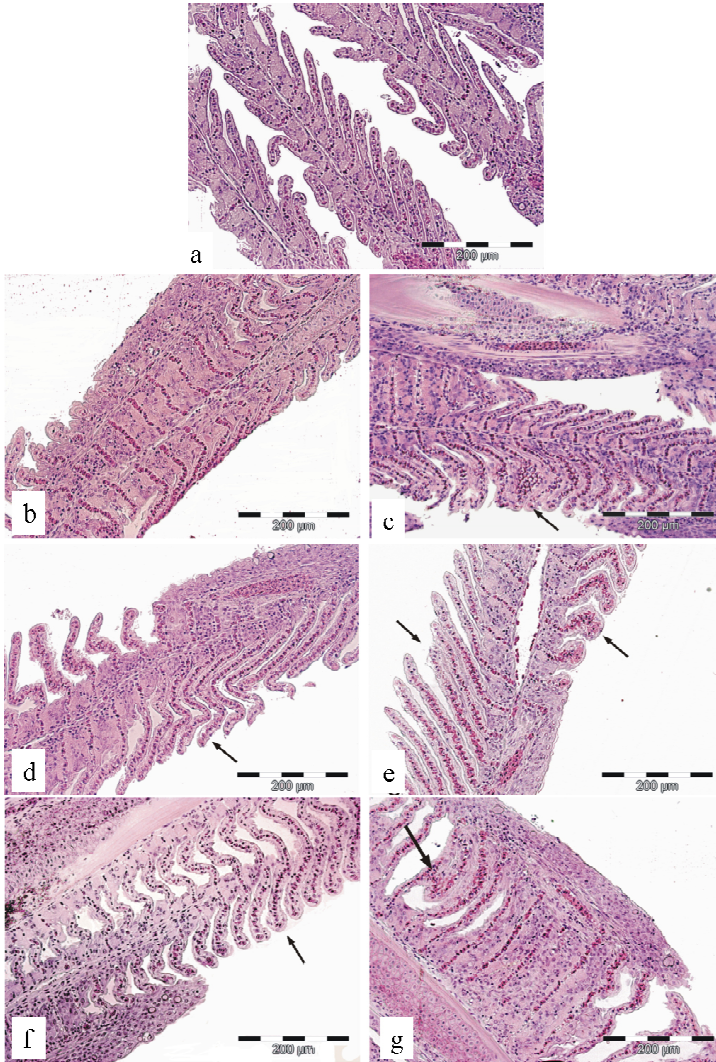
Figure 4:   Gill morphology: (a) sea-water control; (b) solvent control (0.1‰ DMSO); (c) 0.5 mg/L TNT at 24h; (d) 1 mg/L TNT at 6 h; (e) 2.5 mg/L TNT at 6 h; (f) 1 mg/L TNT at 24 h; (g) 2.5 mg/L TNT at 24 h. At 1 mg/L TNT (d), epithelial lifting (arrow) leading to rupture (e) of branchial epithelium (left arrow) and vascular congestion (right arrow) at 2.5 mg/L TNT. After 24 h at 0.5 mg/L TNT (c) oedema and epithelial detachment in secondary lamellae and vascular congestion (arrow). At 1 mg/L TNT (f), severe dilation of lamellar capillaries (arrow). At 2.5 mg/L TNT (g), distortion of lamellae with protrusion (arrow), fusion and obliteration of interlamellar spaces.

distinct TNT metabolites generated after 6 and 24 h. GST in particular is involved in conjugation of electrophilic metabolites, with glutathione playing an important role in protecting tissues from oxidative damage. Therefore the increase in GST gene expression and related enzyme activity also suggests activation of antioxidant defences in TNT-exposed eels.

Regarding neurosteroidogenesis, the downregulation of both P450*scc* and StAR genes at 6 h, suggest the ability of TNT to affect brain steroidogenic pathways by producing an inhibition of the initial phases of neurosteroidogenesis. Both these two proteins are acutely regulated in neurosteroidogenic tissues, making these responses highly effective in acute and short-term exposure scenarios (Lyssimachou and Arukwe [13]). As steroids production is of utmost importance for several physiological and reproductive functions of the organism (Arukwe [14]), these results suggest that the alteration of key proteins in the steroidogenic pathway may be a mechanism involved in TNT impact on fish.

Gill alterations observed in TNT exposed eels are similar to those already reported for environmental contaminants such as OPs, PAHs and Alkylphenol Ethoxylates (APEs) and can be regarded as acute damage associated with short–term exposure (Mallat [15], Brusle [16]). The TNT effects observed in gill of eels suggest a direct correlation between exposure to the compound and gill pathology and highlight a novel aspect of TNT toxicity. Gills histopatologies in fact are often associated with physiological effects such as failure of osmoregulation, acid-base balance and respiration and can significantly affect fish health (Evans [17], Smith *et al* [18], Simonato *et al* [19]).

References

[1] Talmage, S.S., Opresko, D.M., Maxwell, C.J., Welsh, C.J.E., Cretella, F.M., Reno, P.H. & Daniel, F.B., Nitroaromatic munition compounds: environmental effects and screening values. *Reviews of Environmental Contamination and Toxicology*, **161**, pp. 1–156, 1999.

[2] Ek, H., Dave, G., Birgersson, G. & Förlin, L., Acute effects of 2,4,6-trinitrotoluene (TNT) on haematology parameters and hepatic EROD-activity in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Ecosystems Health and Management*, **64**, pp. 415–421, 2003.

[3] Ek, H., Dave, G., Sturve, J., Almroth, B.C., Stephensen, E., Förlin, L. & Birgersson, G., Tentative biomarkers for 2,4,6-trinitrotoluene (TNT) in fish (*Oncorhynchus mykiss*). *Aquatic Toxicology*, **72 (3)**, pp. 221–230, 2005.

[4] Johnson, M.S., Vodela, J.K., Reddy, G. & Holladay, S.D., Fate and the biochemical effects of 2,4,6-trinitrotoluene exposure to tiger salamanders (*Ambistoma tigrinum*). *Ecotoxicology and Environmental Safety*, **46**, pp. 186–191, 2000.

[5] Sims, J.G. & Steevens, J.A., The role of metabolism in the toxicity of 2,4,6-trinitrotoluene and its degradation products to the aquatic amphipod *Hyalella azteca*. *Ecotoxicology and Environmental Safety,* 2007. Doi:10.1016/j.ecoenv.2007.08.019.

[6]   Ownby, D.R., Belden, J.B., Lotufo, J.R. & Lydy, M.J., Accumulation of trinitrotoluene (TNT) in aquatic organisms: Part 1 - Bioconcentration and distribution in channel catfish (*Ictalurus punctatus*). *Chemospher*e, **58**, pp. 1153–1159, 2005.

[7]   Burke, M.D. & Mayers R.T., Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver**.** *Biochemistry and Pharmacology*, **48 (5)**, pp. 923–936, 1994.

[8]   Habig, W.H., Pabst, M.J. & Jakoby, W.B., Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biology and Chemistry*, **249 (25)**, pp. 7130–7139, 1974.

[9]   Collier, A.C., Tingle, M.D., Keelan, J.A., Paxton, J.W. & Mitchell, M.D., A highly sensitive fluorescent microplate method for the determination of UDP-glucoronosyl transferase activity in tissues and placental cell lines. *Drug Metabolism and Disposition*, **28**, pp. 1184–1186, 2000.

[10] Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, pp. 248–254, 1976.

[11] Culling, C.F.A., Allison, R.T. & Barr, W.T., *Cellular pathology technique*. 4th edn. Butterworth: London, 1985.

[12] Reddy, G., Chandra, S.A.M., Lish J.W. & Qualls, C.W., Toxicity of 2,4,6-trinitrotoluene (TNT) in hispid cotton rats (*Sigmodon hispidus*): hematological, biochemical and pathological effects. *International Journal of Toxicology*, **19 (3)**, pp. 169–177, 2000.

[13] Lyssimachou, A., & Arukwe, A., Alteration of brain and interrenal StAR protein, P450scc and CYP11 mRNA levels in Atlantic Salmon after nominal waterborne exposure to the synthetic pharmaceutical estrogen, ethynylestradiol. *Journal of. Toxicology and Environmental Health,* **70(7)**, pp. 606–613, 2007.

[14] Arukwe, A., Modulation of brain steroidogenesis by affecting transcriptional changes of steroidogenic acute regulatory (StAR) protein and cholesterol side chain cleavage (P450scc) in Juvenile Atlantic salmon (*Salmo salar*) is a novel aspect of nonylphenol toxicity. *Environmental Science and Technology*, **39**, pp. 9791–9798, 2005.

[15] Mallatt, J., Fish gill structural changes induced by toxicants and other irritants: a statistical review. *Canadian Journal of Fish Aquatic Science*, **42**, pp. 630–48, 1985.

[16] Brusle, J., The eel (*Anguilla sp*) and organic chemical pollutants. *Science of the Total Environment*, **102**, pp. 1–19, 1991.

[17] Evans, D.H., The fish gill: site of action and model for toxic effects of environmental pollutants. *Environmental Health and Perspectives*, **71**, pp. 47–58, 1987.

[18] Smith, C.J., Shaw, B.J. & Handy, D.R., Toxicity of single walled carbon nanotubes to rainbow trout, (*Oncorhynchus mykiss*): Respiratory toxicity, organ pathologies, and other physiological effects. *Aquatic Toxicology*, **82 (2)**, pp. 94–109, 2007.

[19] Simonato, J.D., Guedes, C.L.B. & Martinez, C.B.R., Biochemical, physiological, and histological changes in the neotropical fish Prochilodus lineatus exposed to diesel oil. *Ecotoxicology and Environmental Safety*, **69(1)**, pp. 112–20, 2008.