Exposure to dissolved TNT causes multilevel biological effects in Baltic mussels (*Mytilus* spp.)

Romina Schuster [a], Jennifer S. Strehse [b], Aino Ahvo [c], Raisa Turja [c], Edmund Maser [b], Ulf Bickmeyer [a], Kari K. Lehtonen [c], Matthias Brenner [a,*]

[a] *Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, Am Handelshafen 12, 27570, Bremerhaven, Germany*
[b] *Institute of Toxicology and Pharmacology for Natural Scientists, University Medical School, Schleswig-Holstein, Brunswiker Str. 10, 24105, Kiel, Germany*
[c] *Finnish Environment Institute, Marine Research Centre, Agnes Sjöbergin katu 2, 00790, Helsinki, Finland*

ARTICLE INFO

ABSTRACT

Baltic mussels (*Mytilus* spp.) were exposed to the explosive trinitrotoluene (TNT) for 96 h (0.31–10.0 mg/L) and 21 d (0.31–2.5 mg/L). Bioaccumulation of TNT and its degradation products (2- and 4-ADNT) as well as biological effects ranging from the gene and cellular levels to behaviour were investigated. Although no mortality occurred in the concentration range tested, uptake and metabolism of TNT and responses in antioxidant enzymes and histochemical biomarkers were observed already at the lowest concentrations. The characteristic shell closure behaviour of bivalves at trigger concentrations led to complex exposure patterns and non-linear responses to the exposure concentrations. Conclusively, exposure to TNT exerts biomarker reponses in mussels already at 0.31 mg/L while effects are recorded also after a prolonged exposure although no mortality occurs. Finally, more attention should be paid on shell closure of bivalves in exposure studies since it plays a marked role in defining toxicity threshold levels.

1. Introduction

After the Second World War, the Allies in Europe were facing a substantial dilemma. They had to dispose the unused weapons that were produced during the war as quickly as possible, and preferably in a cost-efficient way. In 1946, a law authorizing the "disposal of hazardous ammunition by dumping at sea or demolition" was launched (Böttcher et al., 2011), and therefore allowed the dumping of chemical and conventional munition at sea. A vast amount of weapons of all kinds, from cartridge cases to torpedoes and bombs, were disposed in the German coastal waters during the following years. Approximately 1.8 million tonnes of conventional munitions were dumped in the German coastal waters of both the North Sea and the Baltic Sea (Böttcher et al., 2011). In addition to the Second World War, considerable quantities of munitions had been dumped in the North and Baltic Sea already during the First World War, not only through active warfare but also during exercises at sea as well as by the disposal of unusable ammunition (Brenner et al., 2017). Due to the lack of information during the disposal as well as the occurrence that fishermen later on recovered at least 200,000 tonnes of munitions, the exact amount still present in the sea is unknown. Today,

it is estimated for German waters that about 1.3 million and 300,000 tonnes are dumped in the North Sea and the Baltic Sea, respectively (Böttcher et al., 2011). Over the years, water currents and bottom trawling activities have caused the ammunition to spread over substantial distances in the sea, making the exact location of the ordnance difficult (Böttcher et al., 2011; Beldowski et al., 2014).

Until the 1970s, the disposal of military waste in oceans had been a common method of continuous discarding of ammunition in large quantities. The introduction of agreements such as HELCOM (Baltic Marine Environment Protection Commission) and OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic) allowed the disposal only in acute danger situations and the dumping has received growing concern due to its potentially negative impacts on the marine ecosystem (Lima et al., 2011; Böttcher et al., 2011). Most substances used in conventional ammunition are hazardous and have a high ecotoxicological potential being carcinogenic, mutagenic and toxic to reproduction (the so-called CMR substances) (Bolt et al., 2006).

A precise assessment of the corrosion state of all the different munitions is not possible and varies from practically intact to fully corroded (Beddington and Kinloch, 2005). In addition to salinity, hydrographic

parameters such as oxygen content, temperature and current intensities affect the corrosion of the munitions (Koch, 2009). The release of warfare agents in the future cannot be accurately predicted but it is assumed to take place gradually and to be partially widely spread (Böttcher et al., 2011).

At room temperature, 2,4,6-trinitrotoluene (TNT) is a yellow, solid and chemically stable compound with a water solubility of 130 mg/L (Lotufo et al., 2012, 2013). In liquids TNT is transformed and degraded into 2-amino-4,6-dinitrotoluene (2-ADNT) and 4-amino-2,6-dinitrotoluene (4-ADNT) by a nitroreductase (Lima et al., 2011; Lotufo et al., 2013). Most commonly the transformation is performed by bacteria, both under aerobic and anaerobic conditions (Juhasz and Naidu, 2007).

Toxic effects of TNT were first noticed after the First World War (Lima et al., 2011; Lotufo, 2012). In humans it is mainly absorbed through the skin and metabolized in the liver (Johnson et al., 1994; Lima et al., 2011). With a lethal concentration of 14.8 mg/kg body weight in humans, TNT is highly toxic when ingested. Several endpoints, including methemoglobinemia, cataracts, mutagenic metabolites and possible genotoxic effects, have been reported in human studies (Richter-Torres et al., 1995; Sabbioni and Rumler, 2007). Furthermore, TNT is considered as a potential human carcinogen (Bolt et al., 2006). In the marine environment, TNT interacts with sediments, where it has been detected in varying concentrations ranging from trace amounts close to the detection limit up to several mg/kg wet weight (ww) in some cases (Böttcher et al., 2011). Similarly, concentrations between 3.1 mg/L and 3.38 mg/L were measured in the water column close to lumps of hexanite (castable explosive of German Kaiserliche Marine containing 60% TNT and 40% hexanitrodiphenylamine) at the Kolberger Heide in the Baltic Sea coast (Beck et al., 2019) and onshore in the surface waters of dumping areas (Talmage et al., 1999).

Negative impacts of TNT on the survival of different marine organisms have been studied by Nipper et al. (2009), (Lotufo et al., 2010) and Lotufo (2011). LC_{50} values of TNT have been observed to range between 0.98 (the opossum shrimp *Americamysis bahia*) and 19.5 mg/L (the mussel *Mytilus galloprovincialis*) (Nipper et al., 2001; Rosen and Lotufo, 2007b). In addition, Strehse et al. (2017) showed that TNT and its metabolites bioaccumulate in the tissues of mussels collected from ammunition-contaminated areas. Due to tissue bioaccumulation TNT may enter the marine food web, thus presenting a potential risk also to human seafood consumers (Strehse et al., 2017; Appel et al., 2018).

Mussels (Bivalvia) are well-suited as bioindicator organisms and have been used to study pollution in marine ecosystems for more than four decades (Livingstone and Pipe, 1992; Salazar and Salazar, 1995; Brenner et al., 2014; Farrington et al., 2016). They are abundant in temperate seas around the world and resistant to environmental pollution (Strehse et al., 2017). As filter feeders they process several litres of water daily through their gills. In addition to regular food items, such as organic particles and phytoplankton, also bacteria, parasites and chemical pollutants are ingested (Brenner et al., 2014). Genetic studies on mussels in the Baltic Sea have shown that the populations consist of *Mytilus edulis*, *Mytilus trossulus* and hybrids of both species, and therefore the test organism is designated here as *Mytilus* spp. (Stuckas et al., 2017). Especially in the area around Kiel, where the mussels were collected for the present study, hybrid mussels dominate the local population (Stuckas et al., 2017). As a sessile organism, *Mytilus* spp. provides specific information on the degree of contamination in a particular area (Brenner et al., 2014; Beyer et al., 2017). Additionally, they are a significant food source for many marine organisms and birds and thus a mediator of toxic substances within the food web (Strehse et al., 2017).

Although the dumping activities were conducted many decades ago, knowledge about the biological effects of dumped munitions on marine organisms is scarce. Among the low number of publications hardly any is addressing the Baltic Sea brackish water environment and its specially adapted biota. Therefore, we wanted to fill this knowledge gap by conducting experiments with Baltic mussels, reflecting also the specific local conditions such as temperature and low salinity. By using a multi-

biomarker approach, effects of exposure to toxic compounds deriving from the munitions were examined at different biological levels and functions ranging from gene and cellular level responses to behaviour.

2. Materials and methods

2.1. Origin and handling of the model organism

In order to assess the effects and the toxicity of dissolved TNT on brackish water invertebrates we conducted two experiments, an acute 96 h toxicity test and a 21-d test for chronic effects. *Mytilus* spp. purchased from a commercial farm in the Kiel Fjord (Kieler Meeresfarm) were used for the experiments. The mussels were collected 8 d before the experiments started in November 2017. On-site water temperature (9 °C in November - acute toxicity test/3 °C in January - chronic toxicity test) and salinity (18 psµ) were recorded. The mussels (5–7 cm in length) were placed in a cool box filled with aerated ambient sea water and transported directly to the laboratory, where they were kept in a constant temperature room at 9°/3 °C using 20 L aquaria filled with ambient sea water used for the transport. After 48 h of acclimatization the ambient water was removed stepwise by exchanging half of the volume every 24 h with pre-mixed artificial sea water (ASW) with a salinity of 18 psµ (equal to the salinity in the Kiel Fjord). The ASW was produced daily by mixing deionized water and Coral Pro Salt (Red Sea©). The ASW was stored in 75 L containers and precooled before being used for the experiment. Salinity of ASW was checked daily before the water exchange using a salinometer. The mussels were fed every 48 h with 6 mL live phytoplankton (Premium Reef Blend/Sustainable Aquatics©) per aquarium 2 h prior to the water exchange. The mussels were transferred 24 h prior to the start of the exposure into smaller aquaria (15 L) filled with 10 L of ASW.

### 2.2. Origin and fabrication of the TNT solution

A stock solution of 100 mg/L was produced and provided by the German Federal Facility for the Removal and Remediation for Explosives and Chemical Warfare Agents (GEKA, Munster, Germany). A Second World War grenade was sawed open and the solid TNT was scratched off. The TNT crumbs were dissolved using dichloromethane in a volumetric flask. Aliquots were removed from the solution and transferred into 5 L bottles where the solvent was fully removed by evaporation at room temperature. The explosive was re-dissolved in filtered water by stirring at 80 °C to reach a target concentration. The final concentration was verified and corrected if necessary.

2.3. Experimental setup

To test the acute toxicity of dissolved TNT, a 96 h exposure experiment was performed following OECD guidelines (OECD, 2014). The room temperature was set at 9 °C and the light/dark rhythm at 8:16 h according to the ambient conditions at the mussel farm at the time of mussel collection. For each treatment (0, 0.31, 0.62, 1.25, 2.5, 5, and 10 mg/L), three aquaria were filled with 10 L of ASW spiked with the respective exposure concentration. Seven specimens per aquarium were exposed, with one mussel used for chemical and six for biomarker analyses (Fig. 1). The experimental units were supplied with aeration. Water exchange and re-dosing of the dissolved TNT was conducted on a daily basis.

From one aquarium per treatment, three water samples of 1 mL were taken immediately, and then 4 and 8 h after the re-dosing. The water samples were mixed with 1 mL of methanol and stored at −25 °C for chemical analysis. After 96 h the exposure was stopped, the water was discarded, and the mussels were kept cool and dry until dissection.

Since no mortality could be observed during the 96 h acute toxicity test, one out of the three aquaria in the 10 mg/L treatment was kept filled with the spiked ASW. In this way, the acute toxicity test for the
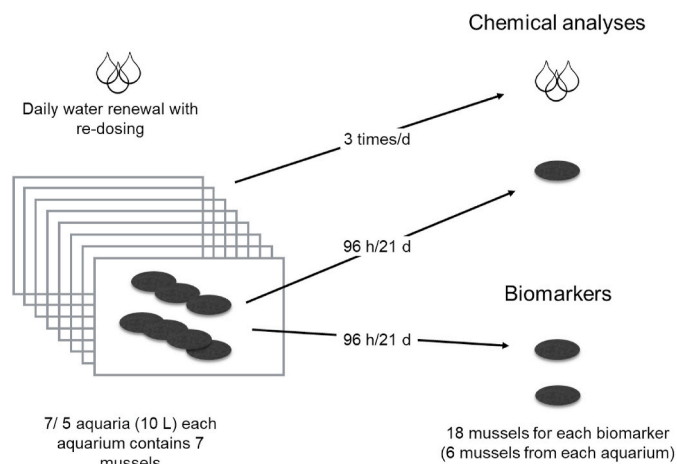
Fig. 1. Experimental setup used for both the 96 h acute and 21 d chronic exposure of Baltic mussels (*Mytilus* spp.) to dissolved TNT. The acute toxicity test was conducted using 6 concentrations (0, 031, 0.62, 1.25, 2.5, 5, and 10 mg/L) and the chronic toxicity test using 4 concentrations (0.31, 0.62, 1.25, and 2.5 mg/L) of dissolved TNT. Per treatment and control, three aquaria, each with seven mussels, were used and sampled thereafter.

remaining mussels was prolonged for 10 days. In addition, another aquaria spiked with a 30 mg/L concentration of dissolved TNT was applied to expose an additional set of mussels. This exposure lasted also for 14 days. During both extensions no daily water exchange was made.

The chronic exposure was carried out for 21 d, also following the OECD guidelines (OECD, 2009). In this setup the four lower concentrations of dissolved TNT that had been applied for the acute test were used, including a control (0, 0.31, 0.62, 1.25, and 2.5 mg/L). Since the chronic exposure experiment was conducted in January, the room temperature was set at 3 °C according to the situation at the time of sampling at the mussel farm. All other experimental conditions and handling processes, including the daily water exchange, were kept identical to the acute exposure scenario described above.

2.4. Behavioural observations

During the 8 h light period the mussels were inspected every 2 h three times per day for gamete release and shell opening, the first check being 2 h after the water exchange and re-dosing in the morning. Aquaria with released mussel eggs or whitish turbidity were denoted as units with spawning events. Shell opening of mussels was determined by counting the share of all mussels with open and closed shells per aquarium and treatment.

During the visual inspection of all the experiments the aquaria were also recorded for dead individuals. Mussels were considered as dead or moribund when their shells were open and they did not respond to touching by closing up. Dead or moribund mussels were immediately removed from the respective aquaria.

2.5. Water and tissue analysis for dissolved TNT

Water concentrations of dissolved TNT in one out of the three aquaria per treatment was monitored during the experiments, resulting in four measurements per concentration and time point after the re-dosing for the acute toxicity test and 21 measurements for the chronic test.

The water samples were diluted with 40 mL of ultrapure water and adjusted to pH 2 with concentrated hydrochloric acid. After this step the samples were pre-concentrated onto SPE columns (Bond Elut ENV, Agilent Technologies, US) and washed with 5 mL of ultrapure water. The analytes were eluted with 2 mL of acetonitrile and re-eluted four times after the first elution. The acetonitrile extracts were analysed with GC/

MS-MS as described in Strehse et al. (2017).

For the tissue analysis of TNT, one mussel per aquarium (three per exposure concentration) were analysed, except for the control and the 10 mg/L concentration where only one individual was analysed. Extraction and analysis of TNT, 2-ADNT and 4-ADNT was carried out as described previously in Strehse et al. (2017) and Appel et al. (2018). In brief, mussels were thawed, placed in a polypropylene tube and homogenized using a T25 Ultra-Turrax with S25N–10G dispersion tool (Ika Works Inc., Staufen im Breisgau, Germany). Per Mussel three aliquots of 1.0 g were taken from the homogenates, extracted with acetonitrile and centrifuged. The supernatants were made up to 10 ml with acetonitrile, followed by GC/MS-MS analysis. Per aliquot two measurements were conducted resulting in n = 6 measurements per mussel. For both water and tissue analyses the TraceGOLD™ TG-5SilMS GC column (30 m × 0.25 mm x 0.25 μm) was used.

2.6. Dissection and morphometric measurements

All tissue samples were taken immediately after the exposure. The mussels were removed from the aquaria and kept cool in labelled plastic containers until further processing. They were dissected in groups of seven individuals (one per treatment) to avoid possible changes in biomarker responses due to delays in the dissection process.

The maximum length of the shell was measured using a Vernier calliper (±0.1 mm) and the mussels were opened by cutting the posterior adductor muscle. The mussels were drained briefly and weighed (ww) before the dissection of gills, digestive gland (two samples) and the mantle tissue. The tissue samples were transferred to conical cryo vials, shock-frozen in liquid nitrogen and stored at −80 °C until further processing. As a last step the empty shells were weighed (ww).

The data obtained were used to calculate the condition index (CI) of mussels using the following formula: CI= (weight soft body [g]/weight shell [g])*100.

2.7. Biomarker analyses

2.7.1. Gene expression

50 mg of the digestive gland tissue was homogenized in 1000 μL of TRIZOL reagent (Invitrogen) with 0.25 mL of 1 mm glass beads (BioSpec) using FastPrep (MP Biomedicals Europe) homogeniser at 6000 rpm for 2 × 25 s. RNA extraction was performed according to the manufacturer's protocol provided with the TRIZOL reagent. The RNA pellet was eluded in 100 μL of RNase-free water, vortexed and heated at 55.5 °C for 3 min to completely dissolve the pellet. The RNA samples were stored at −80 °C until further processing.

The cDNA synthesis was done with SuperScript® III First-Strand Synthesis System for RT-PCR kit (Thermo Fisher Scientific) according to the manufacturer's protocol, using 3 μg of RNA in the reaction. The RNA concentrations were measured with Qubit fluorometer (Invitrogen) using the Qubit RNA-Assay kit (Thermo Fisher Scientific) and cDNA concentrations using the Qubit ssDNA Assay kit (Thermo Fisher Scientific). Quality of the extracted RNA was checked with 2100 Bioanalyzer (Agilent) using the Agilent RNA 6000 Nano Kit according to the manufacturer's protocol.

Gene transcription was analysed with qPCR, done with the Platinum® SYBR® Green kit according to the manufacturer's protocol. The analysed genes were catalase (CAT), superoxide dismutase (SOD) and the tumour suppressor protein p53. Sequences of gene-specific primers used in the qPCR reaction are described in Table 1. The samples were run in three replicates on 96-well qPCR plates with a 7300 Real Time PCR System (AB Applied Biosystem). Total reaction volume in each well was 10 μL, containing 1 μL of undiluted sample (cDNA) and 9 μL of Master Mix according to the protocol, including 0.2 μL of ROX reference dye. Primers were diluted to 10 μM. Standards were prepared by pooling together 2 μL of cDNA from each experiment sample and diluting this pooled sample to 1:1, 1:2, 1:5 and 1:10. Standards were run together

Table 1

Gene-specific primers used in the qPCR reaction. Sequences of forward primer (F) and reverse primer (R) are indicated in the table for each gene.

Primer	Sequence
Catalase-F	5′-AACCGAGAAACTCACCTGAAGGATCC-3′
Catalase-R	5′-ACCTTGGTCAGTCTTGAAGTGGAAT-3′
Superoxide dismutase-F	5′-AGG CGC AAT CCA TTT GTT AC-3′
Superoxide dismutase-R	5′-CAT GCC TTG TGT GAG CAT CT-3′
p53-F	5′-CTAGGTAGACGGGCAGTAGAAGTT-3′
p53-R	5′-GCCTCCTGGTGTTACTGTAGTGAT-3′

with samples in each plate (1 μL of standard and 9 μL of Master Mix per well, all standard dilutions in three replicates). Separate standards were made for the acute toxicity and chronic toxicity experiments.

The cycling program used for CAT and p53 was the following: 50 °C for 2 min hold, 95 °C for 10 min hold, 40 cycles of: 95 °C 15 s, 60 °C for 60 s, melting curve: 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. A similar program was used for SOD, except that 57 °C was used in phase 2. To obtain the relative quantification (RQ) of the gene activity, the first standard curve was calculated from the detected cycle threshold (Ct) values and the cDNA concentrations used (see standard dilutions above). Efficiency (E) was then calculated from the standard curve slope using formula $E = [10^{(-1/slope)}] - 1$. The RQ was calculated for each sample according to the formula $RQ = [(\Delta Ct/E)/sample\ cDNA\ concentration]$, where ΔCt is the difference between sample Ct and mean Ct of the control samples.

2.7.2. Biochemical biomarkers

Digestive gland samples of 12 mussels from each treatment were individually homogenized in 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 10,000 g for 20 min at 4 °C. The supernatants were stored at −80 °C. Gill samples were individually homogenized in 100 mM sodium phosphate (pH 7.0) containing 0.1% Triton-X100 and centrifuged at 10,000 g for 20 min at 4 °C. The supernatants were stored at −80 °C. All homogenizations were performed operating the Tissue-Lyser II (QIAGEN) for 2 × 45 s.

Muscle samples were individually homogenized in 10 mM Tris-HCl buffer containing 5 mM EDTA, 1 mM dithiothreitol (DTT) and 0,1 mM phenylmethylsulphonyl fluoride (PSMF) (pH 7.2), and centrifuged at 16,000 g for 25 min. The supernatants were stored at −80 °C.

All enzyme activity rates were measured in 96-well plates (Greiner) using a microplate reader (Infinite 200, TECAN) and analysed with Magellan software (TECAN). The reaction rate was evaluated according to the best linearity range of the curve. The enzyme activities were adjusted to the protein concentrations of the samples, determined on microplates using the Bradford (1976) method and a bovine serum albumin standard.

2.7.2.1. Acetylcholinesterase (AChE) activity.
AChE activity in the gills was measured according to Bocquené and Galgani (1998), modified for microplates. Briefly, the activity of AChE is measured as the colour change at 412 nm in a mixture containing 0.5 mM DTNB (5,5′-dithiobis 2-nitrobenzoic acid) and 2.6 mM ACTC (acetylthiocholine iodide) at a final concentration in phosphate buffer.

2.7.2.2. Antioxidant defence system (ADS) enzymes.
CAT activity in the gills and digestive gland was measured according to Claiborne (1985) and Vuori et al. (2015). Briefly, the activity is measured as the change in absorbance in a mixture containing 4.3 μM H_2O_2 at a final concentration in phosphate buffer. Glutathione peroxidase (GPx) in the digestive glands was measured according to Vuori et al. (2015), using the commercial kit (Sigma CGP1-1 KT), modified for mussel digestive gland samples. The activity of GPx is measured as the change in absorption at 340 nm in a mixture containing 0.5 mM NADPH, 4.2 mM GSH (reduced glutathione) and 1 unit/mL GR (glutathione reductase) at a final

concentration in a NaN_3 (sodium azide) supplemented reaction buffer. Glutathione reductase (GR) activity in the gill and digestive gland tissues was measured according to Vuori et al. (2015). The activity of GR is measured as the change in absorbance at 340 nm in a mixture containing 1 mM GSSG (oxidized glutathione), 0.75 mM DTNB and 0.1 mM NADPH at a final concentration in EDTA-phosphate buffer (100 mM K-PO_4 + 2 mM EDTA, pH 7.5).

2.7.2.3. Detoxification.
Glutathione *S*-transferase (GST) activity in the gill and digestive gland tissues was measured according to Habig et al. (1974), modified for microplates. GST is measured as the colour change in 340 nm in a mixture containing 2 mM GSH and 1 mM CDNB (1-chloro-2,4 dinitrobenzene) at final concentration in Dulbecco's buffer.

2.7.2.4. Oxidative damage.
Lipid peroxidation (LPX) in the digestive gland was measured according to Vuori et al. (2015). Briefly, the target tissue was homogenized in methanol and centrifuged. A mixture containing sulphuric acid, ammonium ferric sulphate hexahydrate and xylenol orange in methanol was added and the absorbance measured after incubation. Samples were prepared in duplicate with the other sample being treated with TPP (triphenyl phosphate) that blocks lipid hydroperoxides interfering with the analysis. A standard curve was prepared with cumene. LPX concentration was adjusted with tissue wet weight (mg).

2.7.2.5. Metabolic activity.
Enzymatic activities of octopine dehydrogenase (ODH), phosphoenolpyruvate carboxykinase (PEPCK) and pyruvate kinase (PK) were measured in the adductor muscle tissue. The samples were homogenized 1:3 (mg:μL) in homogenization buffer containing 10 mM Tris-HCl, 5 mM Na2EDTA, 1 mM DTT, 0.1 mM PMS at pH 7.2. After homogenization the samples were centrifuged at 15,000 rpm for 25 min at 4 °C, and the supernatant was transferred into new tubes and stored in −80 °C until analysis. ODH activity was measured as reductive condensation of pyruvate and arginine to octopine and water with the oxidation of NADH to NAD + measured at 340 nm, according to the method described in Livingstone et al. (1990) adapted to a half-area microplate. Final concentrations of 100 mM imidazole-HCL buffer, 0.1 mM NADH, 5 mM arginine and 2 mM pyruvate were used in the reaction. Buffer, sample, NADH and arginine were pipetted into the wells and the plate was incubated at 25 °C and shaken for 10 min. Pyruvate was then added into the wells and the measurement was started. Absorbance was measured at 340 nm at 25 °C. PEPCK activity was measured using a commercial kit (Phosphoenolpyruvate Carboxykinase Activity Assay Kit, BioVision Incorporated) according to the manufacturer's instructions adapted to a 96-well half-area plate. Absorbance was measured at 570 nm at 37 °C every 30 s. PK activity was measured using a commercial kit (Pyruvate Kinase Activity Assay Kit, Sigma Aldrich) according to the manufacturer's instructions adapted to a 96-well half area plate. Absorbance was measured at 570 nm at 25 °C every 60 s. Protein concentrations were analysed with a BioRad protein assay using bovine serum albumin as a standard. All measurements were performed with the Infinite 200 PRO plate reader (TECAN).

2.7.3. Histochemical biomarkers

2.7.3.1. Tissue preparation for histochemical biomarkers.
Frozen soft tissue samples were fixed on pre-cooled aluminium chucks for cryostat sectioning. Tissue sections of 10 mm were obtained using a cryotome (Histoserve, Cryo Star NX70) with chamber/knife temperatures of 23/25 °C. The sections were stored at −80 °C until processed for histochemistry and staining.

2.7.3.2. Determination of glycogen, lipofuscin and neutral lipid.
Tissue sections of 6 mussels per aquarium were used to measure glycogen

(GLY), lipofuscin (LIPF) and neutral lipid (NL) levels. For GLY, duplicate sections of the digestive gland were stained using the Periodic Acid-Schiff (PAS) method, modified after Culling (1974). The tissue sections were fixed in Carnoy's fixative for 10 min before being gently rinsed with deionized water. This was followed by an incubation in periodic acid for 10 min and rinsing in deionized water. The sections were then incubated in Schiff's reagent for 5 min and rinsed once more in deionized water. The slides were dehydrated in ethanol solutions (50, 70, 80, 95 and 100%) and cleaned with a xylene replacement (Appliclear). The sections were dried at room temperature and capped with Permount. LIPF accumulation in the lysosomes was determined using the Schmorl's reaction (Pearse, 1985), modified after Moore et al. (2004). The accumulation of NL in cells in the digestive gland was determined using the Oil Red O method, modified after Lillie and Ashburn (1943). More detailed method descriptions of the mentioned staining procedures can be found in Brenner et al. (2014).

2.7.3.3. Image analysis. Tissue sections stained with Schmorl's, Oil Red O and Periodic Acid-Schiff methods were quantitatively and objectively assessed using a computer-assisted image analysis software (Zeiss, AxioVision) combined with a light microscope (Zeiss, Axioskop). The intensity of the staining was determined with a measurement script, whereby a threshold value was determined for the staining products. For the GLY, LIPF and NL assessments, a black-and-white image was generated from a section of the digestive gland tissue at 400-fold magnification. Each section was measured three times resulting in a mean value displayed as area-%.

2.8. Statistics

All biochemical, histochemical and gene expression data were $\log_{10}$ transformed and analysed with ANOVA followed by Tukey's post-hoc HSD test using the SYSTAT 11™ software. All the transformed data were checked for normality of distribution using the Shapiro-Wilk test and for homogeneity of variances using the Kolmogorov-Smirnoff test. The statistical analyses were carried out using Bonferroni correction. For all tests, the significance level was set to $p < 0.05$.

The Integrated Biomarker Response (IBR) index was calculated according to the original protocol of Beliaeff and Burgeot (2002), with modifications presented in Broeg and Lehtonen (2006). Thirteen of the biomarkers applied here were included, excluding PK in which the number of measurements per treatment was only three, and the gene expression biomarkers that were not measured in all the treatment groups. In addition, to further examine the efficiency of the IBR method,

only seven selected biomarkers showing clear differences between the treatment groups were selected, preventing bias by insignificant differences in the calculation procedure. The selection of parameters also balances out the number of the different biological processes investigated, especially reducing the amount of the ADS enzymatic parameters measured from the different tissues (which itself is an important issue regarding the time of exposure and subsequent responses). The biomarkers selected for the 13-parameter "total biomarker response" included GPx, NL, GR, GLY, PEPCK, CAT, LPX, LIPF, and GST measured in the digestive gland, AChE, GR, and CAT measured in the gills, and ODH measured in the muscle. The analysis of a selected set of seven biomarkers included CAT, PEPCK, NL, LPX, GLY, and LIP in the digestive gland, and ODH in the muscle.

3. Results

3.1. Acute toxicity

3.1.1. Mortality

During the 14 d exposure, mortality could only be observed in mussels exposed to 30 mg/L (Fig. 2). The first dead mussel was observed after eight days and after 14 d of exposure all the mussels exposed to 30 mg/L had died. No mortality could be observed in any of the other exposure concentrations.

3.1.2. Release of gametes

Spontaneous spawning of mussels was observed in all tested concentrations. Egg release occurred within the first 48 h in every concentration except for 10 mg/L, where it was seen after 96 h (Table A1). Whitish turbidity indicating sperm release was not detected in any of the aquaria.

3.1.3. Shell closure

In aquaria with a higher concentration of dissolved TNT the mussels were observed to be closed more frequently (Fig. 3). During the whole period, an average of 86% of the control group were observed to be open whereas only approximately 50 and 20% of the mussels were open at 5 and 10 mg/L treatment, respectively. Significant differences were detected comparing mussels exposed to 10 mg/L vs. control ($p < 0.001$), the 0.31 ($p = 0.001$) treatment, and the 1.25 mg/L concentration ($p < 0.05$).

3.1.4. Condition index and gonad status

No significant differences in the CI of mussels could be observed
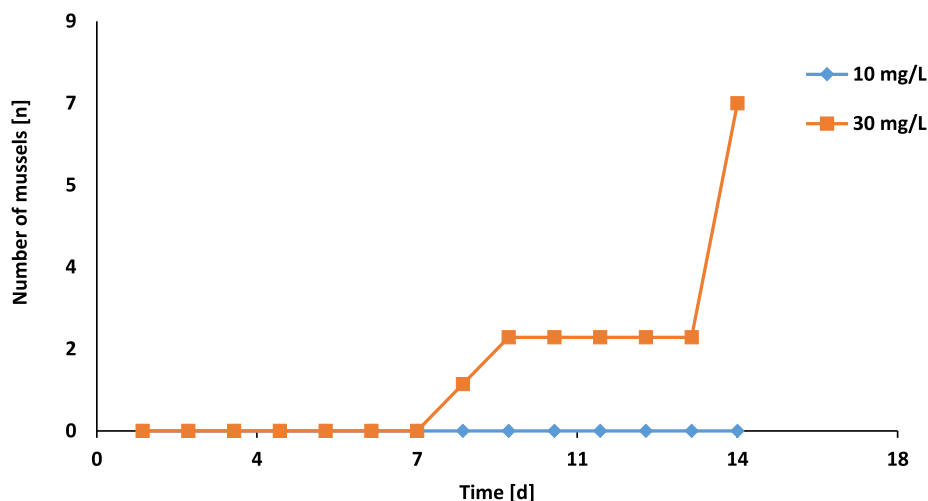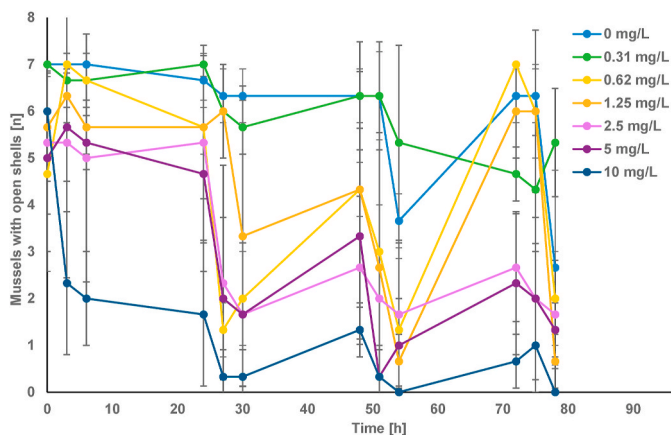

Fig. 2. Mortality of mussels exposed to different concentrations of dissolved TNT during the 96 h (4 d) acute toxicity test, its prolongation up to 14 d and during exposure to an additional TNT concentration of 30 mg/L.

Fig. 3. Number of mussels [mean + SD] with open shells at the different concentrations of dissolved TNT in the 96 h acute toxicity exposure experiment, counted every 2 h during the light period.

between the treatments.

To assess the gonad status mussels were grouped according to "spawned", "gonads exist", "maturing" and "mature" (Fig. A1). If the sex of a specimen could not be defined it was assigned as an individual that may have spawned during the exposure and thus excluded from the biomarker measurements. The sex of most of the individuals could be defined (Table 2).

3.1.5. TNT concentrations in water

Water from one aquarium per treatment was analysed for TNT every day after the daily water exchange and after 4 and 8 h. After 8 h the reduction in the TNT concentration ranged from 5 to 11% (Table 3).

3.1.6. Chemical analysis of TNT and degradation products in tissues

After 96 h of exposure mussel tissues were analysed for the presence of TNT and metabolites. The measured compounds were found in all mussels except for the control group and one mussel from the 0.62 mg/L treatment, where no TNT was detected (Table 4). Of the exposed mussels the lowest amount (21.6 ng/g ww) of TNT was found in the 0.62 mg/L treatment and the highest (1469.9 ng/g ww) in the 10 mg/L treatment. The lowest tissue concentrations of the metabolites 4-ADNT and 2-ADNT (659.3 and 508.8 ng/g ww, respectively) were detected in the 0.31 mg/L treatment and the highest (30872.8 and 25889.9 ng/g ww, respectively) in the 10 mg/L treatment.

3.1.7. Gene expression

Significant differences were observed in the gene expression of CAT and SOD (Table A2 and Fig. 4). For CAT, mussels exposed to 0.31 and 1.25 mg/L showed a higher expression compared to the control group ($F_{2, 26} = 27.941$, both $p < 0.001$). Mussels exposed to 1.25 mg/L showed a higher expression of SOD ($F_{2, 25} = 5.822$, $p < 0.01$) compared to the control group.

Table 2
Number and percentage of male, female and not distinguishable (ND) mussels assessed during the acute toxicity experiment.

TNT Concentration [mg/L]	♂ %	Number of mussels	♀ %	Number of mussels	ND %	Number of mussels
0	44	8	44	8	11	2
0.31	50	9	44	8	6	1
0.62	65	11	18	3	18	3
1.25	50	8	38	6	13	2
2.5	53	9	35	6	12	2
5	53	9	41	7	6	1
10	55	6	36	4	9	1

Table 3
Water concentrations (mg/L) of TNT in the acute toxicity test directly after the daily water exchange ("Start") and after 4 and 8 h. SD = standard deviation; n.d. = not detected.

Exposure group [mg/L]	Measured concentrations mg/L ± SD (% of start conc.)		
	Start	4 h	8 h
0	n.d.	n.d.	n.d.
0.31	0.29 ± 0.04	0.28 ± 0.05 (97)	0.26 ± 0.03 (90)
0.62	0.57 ± 0.06	0.54 ± 0.04 (95)	0.51 ± 0.08 (89)
1.25	1.14 ± 0.11	1.09 ± 0.10 (96)	1.04 ± 0.12 (91)
2.5	2.38 ± 0.09	2.31 ± 0.21 (97)	2.21 ± 0.17 (93)
5	4.66 ± 0.23	4.56 ± 0.36 (98)	4.43 ± 0.41 (95)
10	9.34 ± 0.38	8.89 ± 0.64 (95)	8.68 ± 0.75 (93)

Table 4
Tissue concentrations (mean ± SD) of TNT, 4-ADNT and 2-ADNT measured in mussels in the acute toxicity test. n.d. = not detected.

Exposure group [mg/L]	TNT [ng/g ww]		4-ADNT [ng/g ww]		2-ADNT [ng/g ww]	
	mean	± SD	mean	± SD	mean	± SD
0	n.d.		n.d.		n.d.	
0.31	25.5	12.6	659.3	160.8	508.8	119.1
0.62	21.6	13.1	1287.1	402.7	866.4	348.2
1.25	132.3	23.3	2473.8	606.3	2269.7	443.1
2.5	363.2	396.4	5601.2	1813.4	5154.5	198.2
5	594.0	578.1	7199.4	2846.9	7017.0	1244.1
10	1469.9	115.0	30872.8	977.3	25880.9	967.6

3.1.8. ADS, oxidative damage and neurotoxicity

Significant differences were observed in GPx measured in the digestive gland and CAT in the gill tissue (Table A2 and Fig. 5). For GPx, ANOVA showed a significant difference ($F_{6, 71} = 2.966$, $p < 0.05$) with a lower mean activity in the exposure concentration of 1.25 mg/L ($p < 0.005$) compared to the control group. For CAT in the gill tissue, significant variability ($F_{6, 74} = 3.602$, $p < 0.01$) was recorded, caused by the higher activities in the two lowest exposure concentrations (0.31 and 0.62 mg/L) compared to mussels exposed to 2.50 mg/L (both $p < 0.05$). The levels of LPX were significantly elevated ($F_{6, 74} = 5.580$) in mussels exposed to 0.62 ($p < 0.01$), 1.25 ($p = 0.01$), 2.5 ($p < 0.001$) and 5.0 mg/L ($p < 0.001$) compared to the control group while differences were also observed between treatments 1.25 ($p < 0.05$), 2.5 ($p < 0.12$) and 5.0 mg/L in comparison to the group exposed to 10.0 mg/L ($p < 0.05$). No differences in AChE activity could be observed between the treatments.

3.1.9. Metabolic enzymes

Significantly elevated activities of PK were recorded in mussels exposed to 0.62 and 5.0 mg/L, compared to the control group ($F_{6, 16} = 5.526$, $p = 0.003$; $p = 0.016$ and 0.008, respectively). Differences between mussels exposed to 0.62 and 1.25 mg/L, and 1.25 and 5.0 mg/L were also significant (both $p < 0.05$). For PEPCK, all exposures caused significant elevations in activity ($F_{6, 16} = 14.067$, $p < 0.001$) with a significance level of $p < 0.001$ for all test pairs except for control vs. 0.062 mg/L ($p < 0.01$). Also, mussels exposed to 0.62 and 1.25 mg/L showed higher levels than those exposed to 5.0 mg/L (both $p < 0.05$). ODH activity was significantly ($F_{6, 16} = 4.166$) elevated in mussels exposed to 0.31 ($p < 0.05$), 0.62 ($p < 0.01$), 2.50 ($p < 0.01$) and 5.0 mg/L ($p < 0.05$), compared to the control group (Table A2 and Fig. 6).

3.1.10. NL and LIPF

The levels of NL varied among the different treatment groups. Although no significant differences could be found, NL levels at 0 and 0.31 mg/L seem to be slightly higher compared to the other treatment groups. (Fig. 7).

In contrast, LIPF levels were generally higher when the mussels were exposed to higher TNT concentrations, with more than twice as much
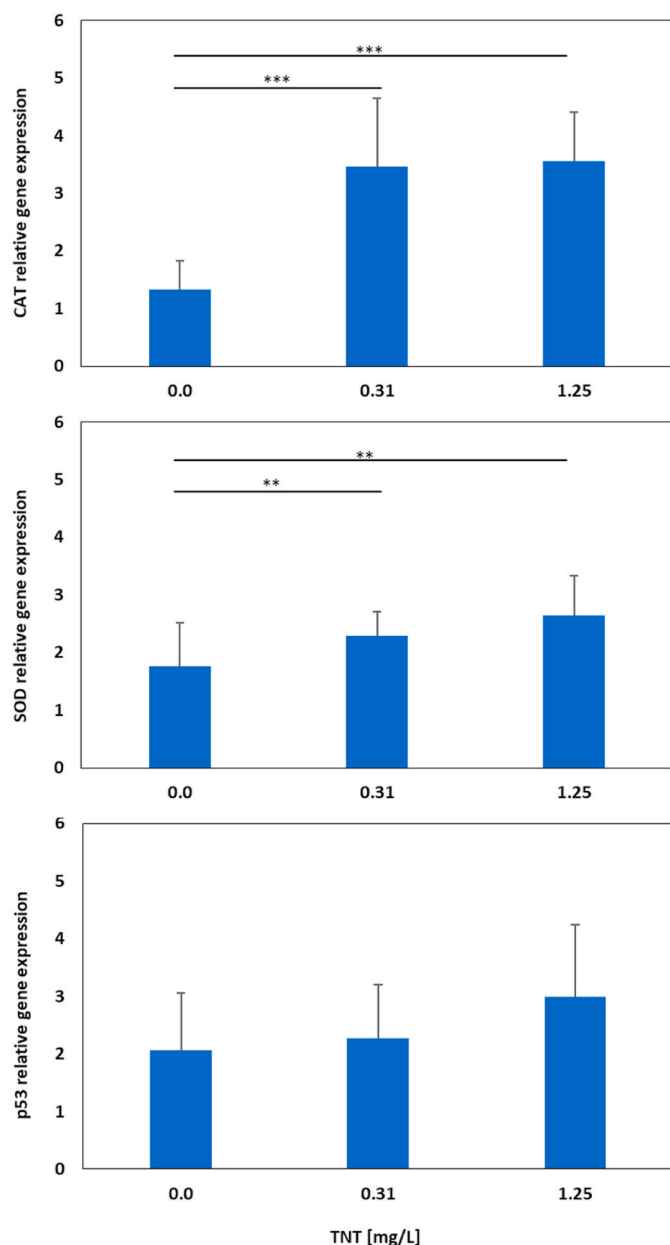
Fig. 4. Gene expression (mean +SD) of CAT, SOD and p53 in the control group (0.0) and mussels exposed to 0.31 and 1.25 mg/L dissolved TNT in the acute toxicity experiment. Significant differences to the control group are given as ** = $p < 0.01$. Differences between the other treatment groups are given in Table A2.

lipofuscin in the 10 mg/L treatment group than in the control group (Fig. 7). Significant differences ($p < 0.001$) were also detected between the treatment groups 0.31 and 1.25 mg/L, 0.62 and 1.25 mg/L and 1.25 and 10 mg/L (Table A2).

3.1.11. GLY
The level of GLY in the digestive gland was significantly higher ($p < 0.01$) in the control group compared to the 1.25 mg/L treatment (Fig. 8). Significant differences were also observed between the 0.62 and 1.25 mg/L treatments ($p < 0.01$) and the 0.62 and 2.50 mg/L treatments ($p < 0.05$).

3.2. IBR

The profiles of the integrated response at the different exposure

concentrations were very similar regardless of the number of biomarkers applied in the calculation (Fig. 9). However, the differences between the treatments were more evident using only the seven selected biomarkers. The integrated stress level of unexposed mussels was clearly the lowest, while exposure to the two lowest concentrations (0.31 and 0.62 mg/L) produced a high response, and in the 7-biomarker assessment also in the second highest concentration (5.00 mg/L). Compared to these the stress level of mussels exposed to the highest concentration (10 mg/L) was lower in both assessments. IBR in the two intermediate exposure concentrations (1.25 and 2.50 mg/L) was lower than in the low and high concentrations especially in the case of the 7-biomarker assessment.

3.3. Chronic toxicity

3.3.1. Observations on mortality, gamete release and shell opening
No dead mussels were found in any of the exposure treatments. In contrast to the acute toxicity test, no spawning was observed. Similar to the acute toxicity test, the mussels exposed to higher concentrations of TNT kept themselves closed more frequently than those exposed to the lower ones (Fig. 10). Significant differences were detected comparing ($F_{4, 100} = 9.5001$) the control to mussel exposed to 0.31 mg/L ($p < 0.05$), 0.62 ($p < 0.001$), 1.25 mg/L ($p < 0.001$), and to the highest concentration of 2.5 mg/L ($p < 0.001$).

3.3.2. Condition index and gonad status
No significant differences in the CI of mussels could be observed between the treatments.

Although no spawning was observed during the experiment, premature individuals were classified as those which might have spawned during the exposure experiment and were therefore removed from further evaluations. Overall, the largest part of the mussels was in a mature state (Tab. A3). In all concentrations, more than 50% of the mussels were maturing or mature, with the largest number of mature individuals in the 0.31 mg/L treatment. Only one mussel exposed to 2.5 mg/L was immature and therefore excluded from the biomarker assessment.

3.3.3. TNT concentrations in water
After 8 h, the reduction in the TNT concentration ranged from 6 to 12% (Table 5).

#### 3.3.4. Chemical analysis of TNT and degradation products in tissues
The tissues of two mussels per treatment and one from the control group were analysed for TNT and the metabolites 2-ADNT and 4-ADNT. TNT, 2-ADNT and 4-ADNT were found in all the analysed mussels except for the control group. The highest tissue levels of the compounds were found in the 2.5 mg/L treatment with 126.5, 4288.0 and 5247.1 ng/g ww, respectively (Table 6). The lowest TNT, 2-ADNT and 4-ADNT concentrations were measured in the 0.31 mg/L treatment with 30.2, 313.9 and 622.7 ng/g ww, respectively. .

3.3.5. Biomarker gene expression
Significant differences were observed only in the gene expression of p53 (Table A4 and Fig. 11), with elevated expression in mussels exposed to 2.50 mg/L compared to the control group ($F_{2, 24} = 4.999$, $p < 0.05$).

3.3.6. ADS, oxidative damage and neurotoxicity
In the digestive gland, a significant difference ($F_{4, 54} = 5.736$, $p < 0.001$) in GPx activity was observed between the control group and mussels exposed to concentrations of 0.62 and 2.50 mg/L ($p < 0.01$ and $p < 0.05$, respectively), and also between the two lowest exposure concentrations of 0.31 mg/L and the 0.62 mg/L ($p < 0.05$) (Table A4 and Fig. 12). A lower GR activity was recorded in the group exposed to 0.62 mg/L compared to the control ($F_{4, 55} = 3.3.034$, $p < 0.05$). A significantly elevated GST activity was observed in the two highest concentrations of 1.25 and 2.50 mg/L compared to the non-exposed mussels
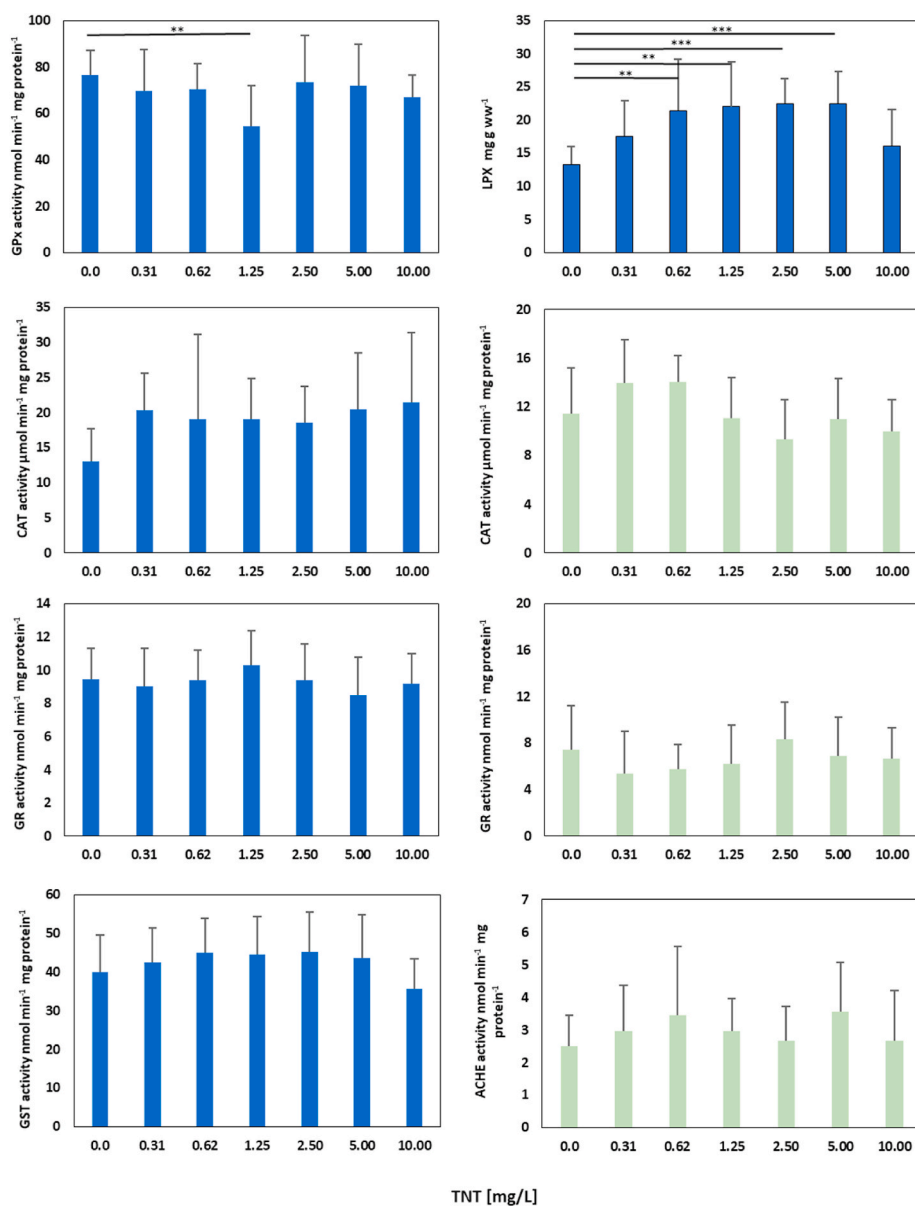
Fig. 5. Activities (mean +SD) of GPx, CAT, GR, GST and AChE in the different exposure concentrations of dissolved TNT in the acute exposure experiment. Blue bars: digestive gland, green bars: gill tissue. Significant differences to the control group (0.0) are given as * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$. Differences between the other treatment groups are given in Table A2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

($F_{4, 55} = 5.231$, $p < 0.01$ and $p < 0.05$, respectively), and also in the 1.25 mg/L concentration compared to the lowest concentration of 0.31 mg/L ($p < 0.05$). Finally, a significantly decreased CAT activity was recorded in the gill tissue of mussels exposed to 0.62 mg/L compared to the control group ($F_{4, 70} = 3.753$, $p < 0.01$). No differences in AChE activity could be observed between the treatments.

3.3.7. Metabolic enzymes

PEPCK showed significant elevations in all exposure groups ($F_{4, 20} = 25.785$, $p < 0.001$), with a difference at $p < 0.001$ for the concentrations of 0.31, 0.62 and 2.50 mg/L, and $p < 0.05$ for 1.25 mg/L compared to the control group (Table A4 and Fig. 13). In addition, differences in PEPCK activity were observed between 0.31 and 1.25 mg/L ($p < 0.05$), 0.62 and 1.25 mg/L ($p < 0.001$), and 1.25 and 2.50 mg/L ($p < 0.001$) exposure groups. ODH showed significant variability ($F_{4, 40} = 5.932$, $p < 0.001$), being significantly elevated in the control group compared to the 0.31 mg L group ($p = 0.012$), and also in mussels exposed to the lowest TNT concentration of 0.31 mg/L compared to the 0.62 ($p = 0.62$), 1.25 ($p < 0.05$) and 2.5 mg/L ($p < 0.001$) exposure groups.

3.3.8. NL and LIPF

Significant differences ($p < 0.001$) were found in the accumulation of NL in the cells of the digestive glands between control and the 1.25 and 2.5 mg/L treatment groups (Fig. 14, Table A4). Concerning LIPF, a similar trend could be observed but the high individual variability resulted in non-significant differences between the treatments.

3.3.9. GLY

The level of GLY in the digestive gland was significantly lower ($p < 0.05$) in the control group compared to the 0.62 mg/L treatment (Fig. 15 and Table A4).

3.4. IBR

Similar to the acute exposure experiment, the profiles of the integrated response at the different concentrations were similar in the 7- and 13-biomarker calculations (Fig. 16). However, the profile in general was very different, with the lowest total stress values remarkably lower in the lowest exposure concentrations (0.31 and 0.62 mg/L) compared to the medium exposure concentrations (1.25 and 2.50 mg/L). The 13-
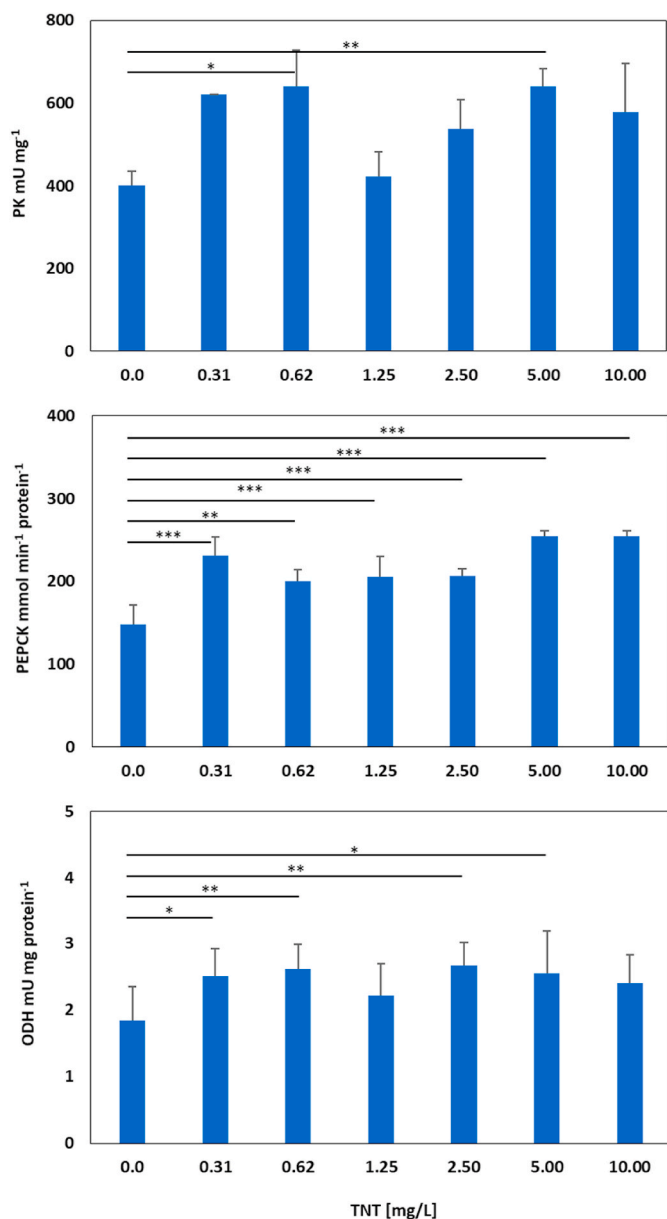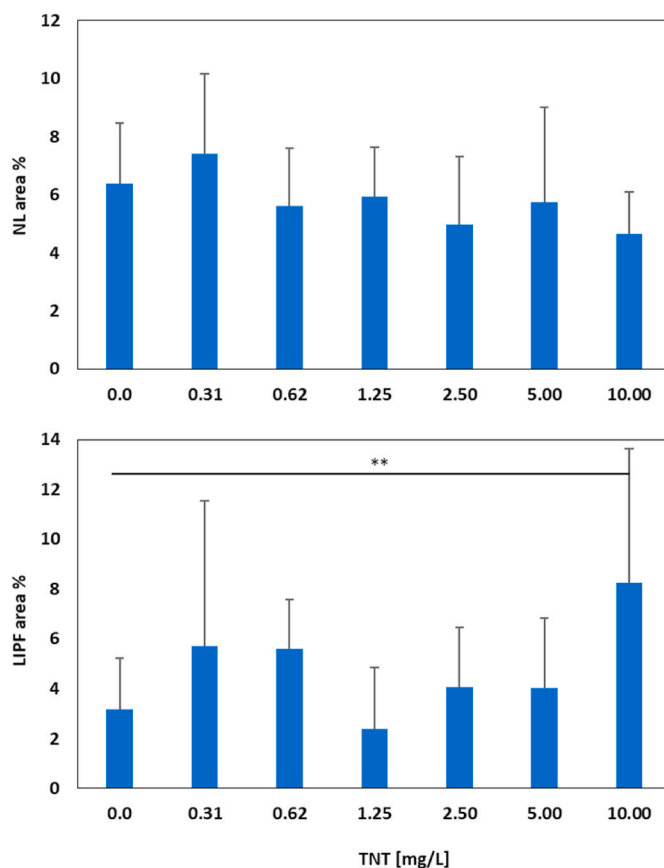
Fig. 6. Activities (mean +SD) of PK, PEPCK and ODH in the different exposure concentrations of dissolved TNT in the acute exposure experiment. Significant differences to the control group (0.0) are given as * = p < 0.05, ** = p < 0.01 and *** = p < 0.001. Differences between the other treatment groups are given in Table A2.

biomarker assessment produced a markedly higher stress level in the control group compared to the use of 7 biomarkers in the calculation.

4. Discussion

The negative effects of TNT on the health of humans and terrestrial organisms have been shown in several studies (Johnson et al., 1994; Lima et al., 2002; Saka, 2004). Also for marine species several laboratory experiments showed that explosives are taken up, metabolized and accumulated in different marine species like blue mussels (*M. galloprovincialis*), sheepshead minnow, bladder wrack (*Fucus vesiculosus*), Atlantic salmon and others (Mariussen et al., 2018; Lotufo et al., 2016; Ballentine et al., 2015; Rosen and Lotufo, 2007a). Data of lethal and sublethal effects of TNT, its metabolites 2-ADNT and 4-ADNT as well as for other explosive chemicals are known for different marine

Fig. 7. Levels of NL and LIPF (area %, mean +SD) in the cells of the digestive gland tissue in the different exposure concentrations of dissolved TNT in the acute toxicity test.
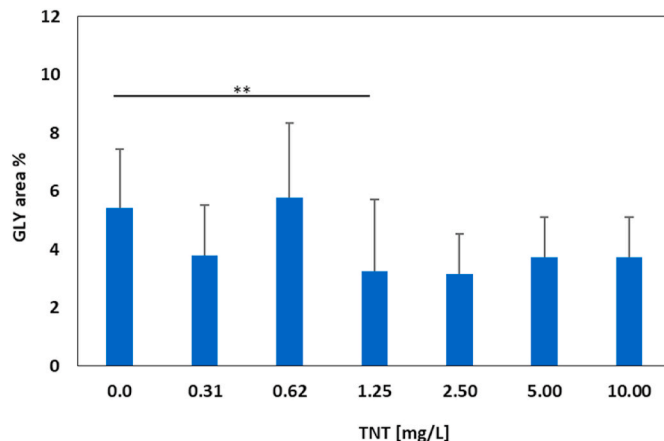
Fig. 8. Concentrations of GLY (area %, mean +SD) in the cells of the digestive gland in the different exposure concentrations of dissolved TNT in the acute toxicity test. A significant difference to the control group (0.0) is given as ** = p < 0.01. Differences between the other treatment groups are given in Table A2.

species. For instance, Nipper et al. (2001) have proven that TNT, tetryl and hexogen (RDX) have negative effects on different marine species such as algae, polychaete, opossum shrimp and redfish. The lowest effect concentrations were observed within a range of 0.26–7.6 mg/L. Further, differences were described in the observed toxic effect concentrations of explosives between juvenile and adult marine specimen. Rosen and Lotufo (2007b) for example showed that the embryo-larval development of the Mediterranean mussel *M. galloprovincialis* is impaired already at
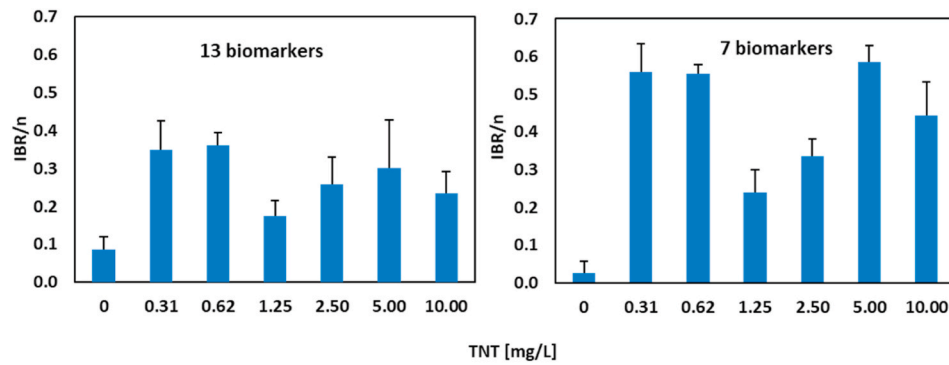
Fig. 9. Integrated Biomarker Response index (IBR) divided by the number of biomarkers used in the calculation (IBR/n) in the different exposure concentrations in the acute exposure experiment using 13 or 7 biomarkers for the calculation. Mean +SD (vertical bars) calculated from different data arrangements.
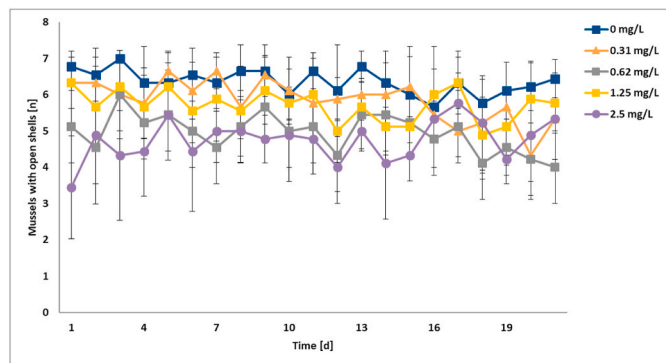

Fig. 10. Number of mussels with open shells at the different concentrations of dissolved TNT in the 21 d chronic toxicity exposure experiment, counted every 2 h during the light period.

Table 5
Water concentrations (mg/L) of TNT in the chronic toxicity test directly after the daily water exchange ("Start") and after 4 and 8 h. SD = standard deviation; n.d. = not detected.

	Measured concentrations mg/L ± SD (% of start conc.)		
Exposure group [mg/L]	Start	4 h	8 h
0	n.d.	n.d.	n.d.
0.31	0.28 ± 0.06	0.27 ± 0.06 (96)	0.25 ± 0.09 (89)
0.62	0.59 ± 0.08	0.57 ± 0.07 (97)	0.52 ± 0.11 (88)
1.25	1.16 ± 0.15	1.11 ± 0.12 (96)	1.07 ± 0.16 (92)
2.5	2.34 ± 0.21	2.29 ± 0.25 (98)	2.20 ± 0.30 (94)

Table 6
Tissue concentrations mean ± SD) of TNT, 4-ADNT and 2-ADNT measured after the experiment in mussels in the chronic toxicity test. n.d. = not detected.

	TNT [ng/g ww]		4-ADNT [ng/g ww]		2-ADNT [ng/g ww]	
Exposure groups [mg/L]	Mean	± SD	mean	± SD	mean	± SD
0	n.d.		n.d.		n.d.	
0.31	30.2	4.9	622.7	129.7	313.9	49.3
0.62	41.4	20.2	1659.6	64.5	1170.4	125.5
1.25	49.3	19.1	1290.6	774.7	917.9	658.9
2.5	126.5	33.2	5247.1	266.5	4288.0	1874.9

very low concentrations, whereas e.g. the lethal concentrations for adult mussels were much higher.

However, much less is known when it comes to brackish water

systems such as the Baltic Sea. A recent 93-day field caging study at Kolberger Heide, a major munition dumping site on the German Baltic Sea coast, reported that the exposed mussels showed a decrease in shell growth in combination with weight loss (Strehse et al., 2017). However, the experimental setup used did not allow for a correlation between effects and the presence of TNT. For this reason, the present study was created, to elucidate whether the exposure to dissolved TNT can be correlated to negative biological effects measured in the deployed specimen.

In order to avoid stress caused by reproduction, the experiments took place in late autumn and thus outside the known reproduction period (April and August–September). Nevertheless, spawning was observed in all concentrations during the acute TNT exposure. Histological examinations revealed that most of the mussels were sexually mature. By excluding pre-mature individuals and specimens that obviously had spawned it was ensured that all test organisms were in a comparable state of reproduction. In addition, evaluation of the CI confirmed that all animals were also in a similar physiological status, therefore comparable, and thus the differences observed after the exposition are most probably correlated by the TNT exposure.

The exposure concentrations for the acute toxicity test were selected according to maximum values measured in the field at Kolberger Heide. Here, Beck et al. (2019) detected 3.1 μg/L dissolved TNT in a water sample collected very close to a lump of hexanite a widely used military explosive with high percentage of TNT.

During both tests TNT in the exposure water was monitored at the time of re-dosing and 4 and 8 h thereafter, showing -although not significant-lower TNT concentrations in the exposure water after 8 h in both experiments. This is correlated with an increase of TNT and especially 2- and 4-ADNT in the mussel tissues measured after the experiments.

In the present study the mussels were exposed to dissolved TNT at low temperatures, as measured at the day mussel were sampled at the Meeresfarm for the respective lab experiment. Further, artificial room light and artificial seawater was used. After the exposures, TNT, 4-ADNT and 2-ADNT were found in mussel tissues in relation to the nominal concentrations of TNT in the different treatments. Although others sources of the ADNTs, such as impurities of the stem solution, or bacterial growth on the mussels shells cannot be excluded, it remains quite likely that the mussel themselves metabolite the TNT to 2- and 4-ADNT as also proven by Rosen and Latufo (2007 a,b) for the closely related Mediterranean mussel *M. galloprovincialis*.

Further, Rosen and Lotufo (2007b) also showed that the mussel *M. galloprovincialis* responds with behavioural changes to pollutants in the water column. One of these responses is the spontaneous release of gametes from mussels with mature gonads. One possible interpretation of the release is that mussels under an acute deterioration of environmental conditions choose the rapid drifting of gametes into areas with better conditions. The release of gametes occurred also in the present
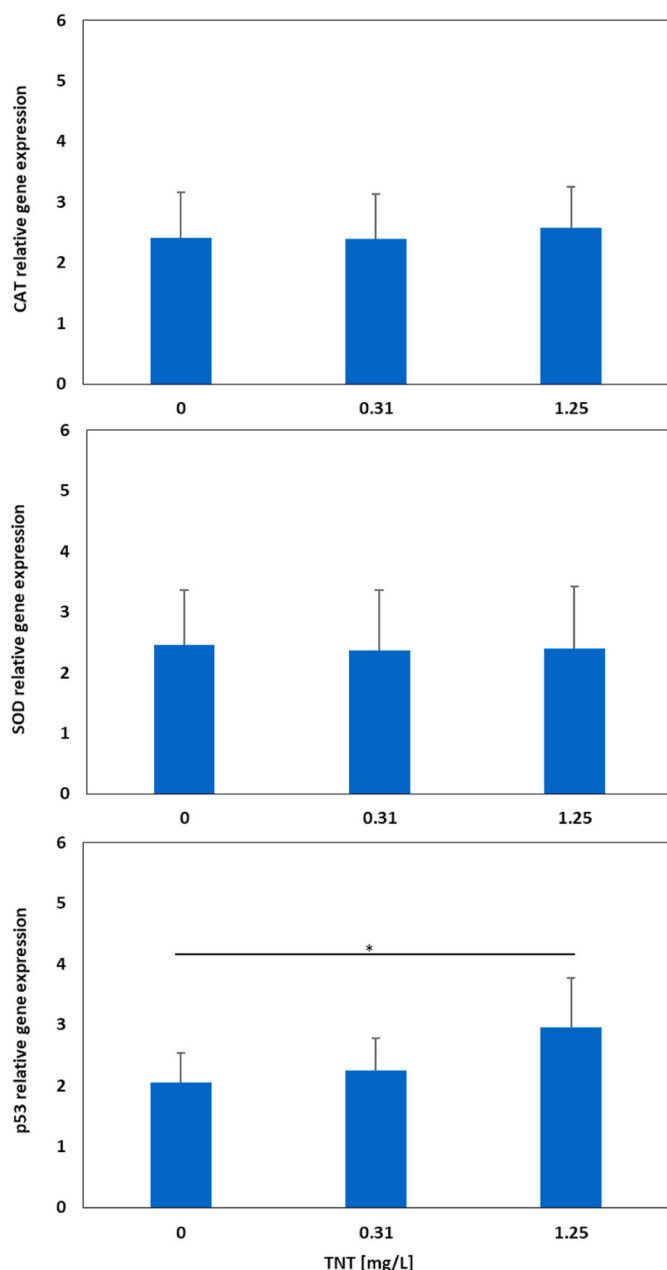
Fig. 11. Gene expression (mean +SD) of CAT, SOD and p53 in the control group (0) and mussels exposed to 0.31 and 1.25 mg/L in the chronic toxicity experiment. Significant difference to the control group is given as * = p < 0.05. Differences between the other treatment groups are given in Table A4.

study, but only during the acute toxicity test. Here, the gamete release was denoted in all treatments including the control, indicating that the spawning was not specifically due to TNT contamination but could also have been triggered by other stress factors such as laboratory conditions in general or due to the handling stress of mussels while being prepared for the experiment.

In the present study, the number of mussels recorded having closed valves during the exposure depended on the concentration of TNT, and this potentially affected its uptake in the different treatments. Such a simple but effective behaviour is known also from exposures to other toxicants and has been described in several other studies (Viarengo and Canesi, 1991; Landsberg, 2002; Fernández-Reiriz et al., 2008; Bianchia et al., 2019; Bickmeyer et al., 2020). Rosen and Lotufo (2007b) investigated *M. galloprovincialis* and calculated LC 50 values of 19.5 mg/L of dissolved TNT for adult individuals. Similar to their experiments,

lethality occurred in our study only at the 30 mg/L exposure concentration. However, due to the sharp increase in mortality rates towards the end of the exposure it can be assumed that the actual LC_{50} value of TNT is lower for the mussels of the present study.

Furthermore, Rosen and Latufo (2007b) did not record shell closure of their mussels, assuming, however, that the mussels showed normal filtering activity. Based on this the authors concluded an overall low toxicity of TNT for mussels. In contrast, the results from our experiments showed that mussels reduce significantly shell opening under high TNT concentrations, which may explain the low mortalities even when exposed to high concentrations of dissolved TNT indicating the efficiency of shell closing as a potent defence mechanism. Thus, in short-term toxicity testing it should be considered that mussels can remain closed even throughout the whole exposure period, likely resulting in a biased toxicity assessment. Overall, different behavioural pattern of species under the exposure of contaminants should be carefully taken into account when assessing toxicities for different species.

Significant responses were observed at the biochemical and cellular biomarker level, some of them apparently closely linked with observations on the shell closure behaviour of mussels. The ADS was activated during acute exposure to low TNT concentrations with elevated gene expression of CAT and SOD in the digestive gland at 0.31 and 1.25 mg/L and higher CAT activity throughout the whole concentration range compared to the control group. However, the significantly elevated LPX levels in almost all exposure concentrations compared to the control group demonstrate that neutralization of the elevated levels of reactive oxygen species (ROS) resulting from the metabolism of TNT was not sufficient and membrane damage took place during the exposure. A notable observation is that LPX was not elevated at the highest concentration of 10 mg/L, possibly related to the significantly increased shell closure at this concentration. In the chronic exposure, however, the situation was completely different with no elevations in the gene expression of neither CAT nor SOD and a coinciding significant decrease in the activities of CAT in the gills at 0.62 mg/L, and in GPx at 0.62 and 2.50 mg/L, and GR at 0.62 mg/L in the digestive gland. This can be interpreted as "bell-shape" responses of these ADS biomarkers due to prolonged stress caused by the TNT exposure and not compensated by the ADS. The significantly elevated activity of GST at the two highest concentrations of the chronic exposure (1.25 and 2.50 mg/L) indicate uptake and active detoxification of TNT at these concentrations, constantly requiring additional metabolic energy.

In the acute toxicity experiment, the metabolic enzymes PK, PEPCK and ODH showed elevated activities in most of the TNT treatments compared to the control group, indicating markedly increased energy needs (PK and PEPCK) and a shift towards additional energy production from anaerobic metabolism (ODH). In the chronic exposure the situation had turned around, with lowered PEPCK activities observed in most TNT treatments compared to the control group, indicating a general depression in aerobic metabolism under chronic exposure. Regarding ODH, the general significant elevation in the activity, notably also in the control group, indicates the increasing effects of laboratory maintenance on the physiology of the mussels during the chronic experiment. This is indeed observed also in the elevated level of PEPCK in the control group compared to the acute toxicity experiment, probably signifying the inability of the individuals exposed to TNT respond to the increased energy needs created by the prolonged laboratory maintenance period.

Accumulation of the metabolic end-product LIPF in the lysosomes of the digestive gland is regarded as a result of oxidative stress (Viarengo and Nott, 1993). With increasing TNT concentrations, the amount of LIPF varied substantially during the acute toxicity test with only the 10 mg/L treatment producing a significant increase in the LIPF concentration. In contrast, in the chronic exposure signs of a trend of increasing LIPF levels could be seen, as reported in other studies in the presence of organic pollutants (Au et al., 1999; Au, 2004). However, this trend was not confirmed by statistics and differences were not significant between the treatments.
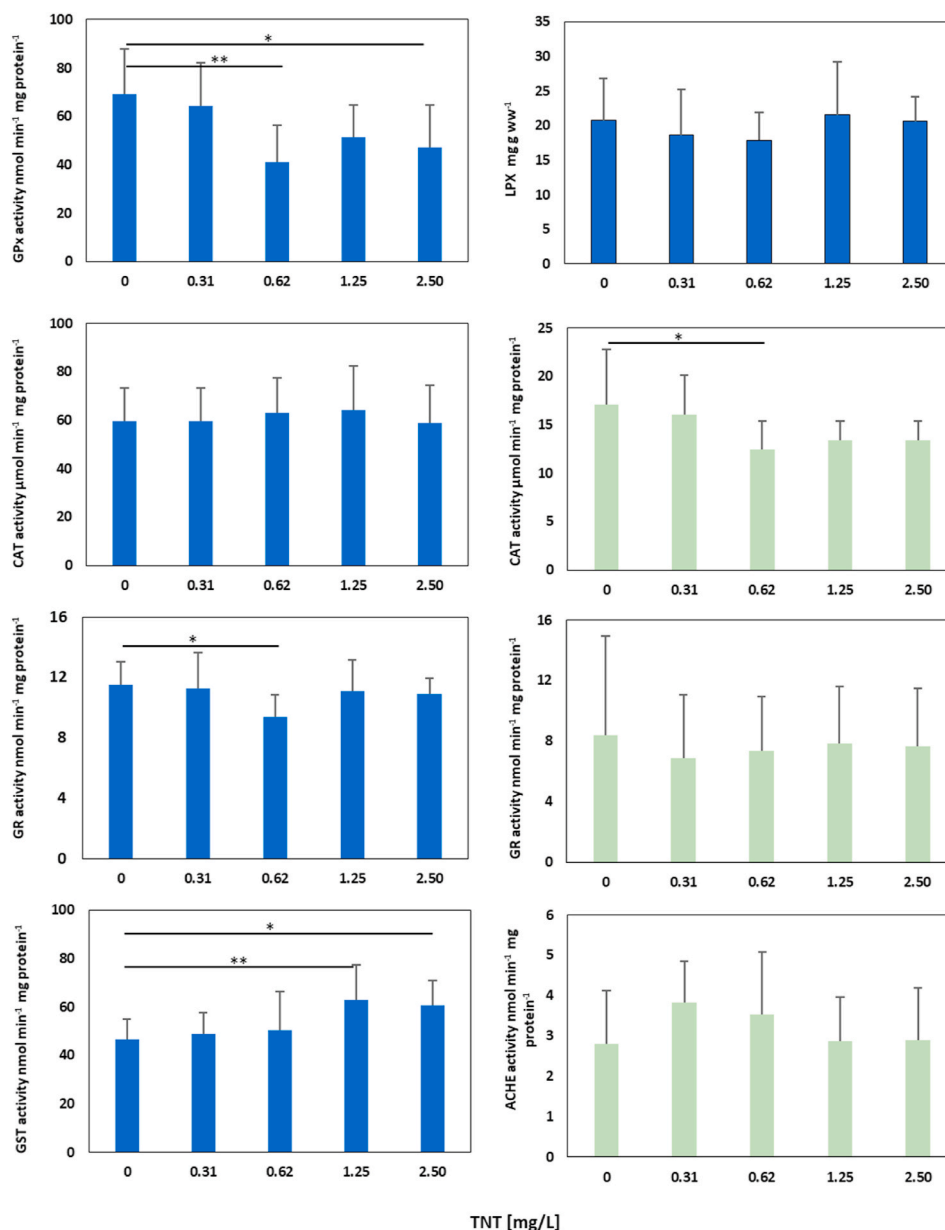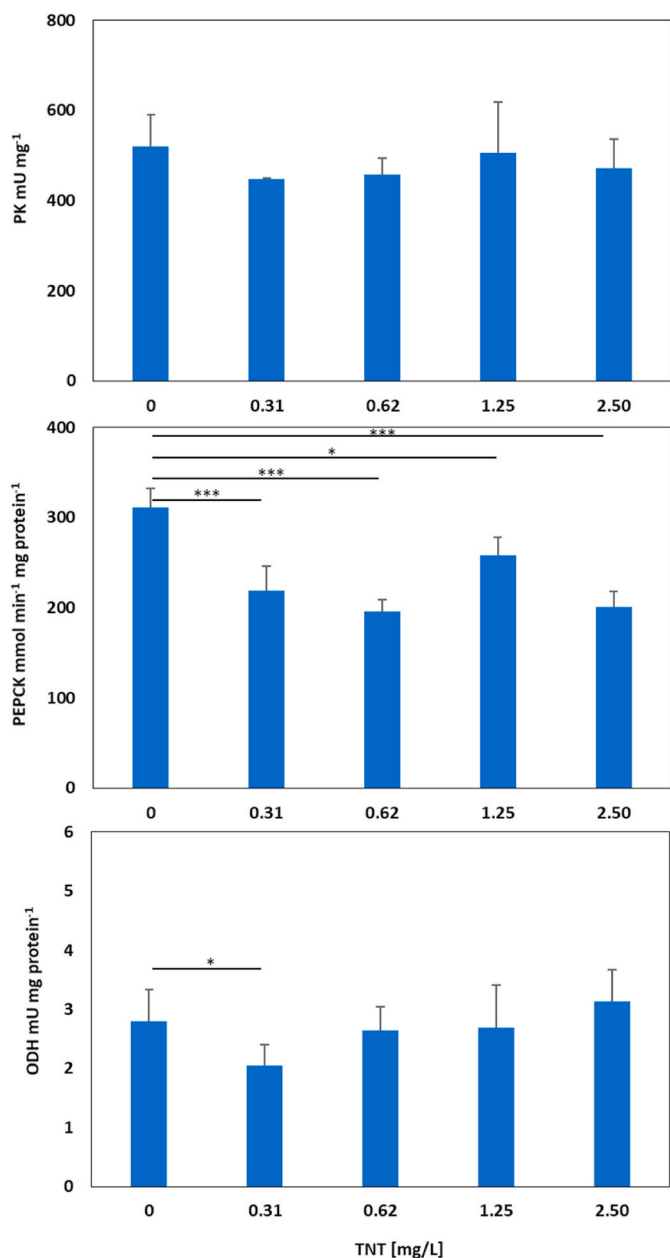
Fig. 12. Activities (mean +SD) of GPx, CAT, GR, GST and AChE in the different exposure concentrations of dissolved TNT in the chronic exposure experiment. Blue bars: digestive gland, green bars: gill tissue. Significant differences to the control group (0) are given as * = p < 0.05 and ** = p < 0.01. Differences between the other treatment groups are given in Table A4. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

An increase in NL is an indicator of lipidosis, i.e. an unnatural accumulation of lipids in tissues and cells, which is involved in the induction of apoptosis (Lowe et al., 1981; Sun et al., 2007; Brenner et al., 2014). Here, the increased accumulation of NL coincided with higher TNT concentrations in the chronic exposure but not in the short-term exposure; the latter may be due to a slower response time or more efficient shell closure during the short-term exposure.

GLY is the primary energy reserve in most bivalves (Patterson et al., 1999). Numerous environmental factors including chemical pollution increase stress in organisms, often leading to increased metabolism and to reduced GLY stores. Here, a lower amount of GLY was expected in mussels exposed to higher TNT concentrations but this could not be confirmed. Possibly, mussels reduce their energy consumption during a longer shell closure period and the GLY stores become depleted more slowly.

During the experiment the higher TNT exposure concentrations led to increased shell closure, which is a common phenomenon in bivalves.

However, the threshold levels for this behaviour can be rather individual and not very sharp, which can be observed from the data obtained. Due to the shell closure of mussels in the applied exposure concentration range the responses did not follow the traditional elevating dose/concentration-response curve. Instead, the response dynamic was regulated also by other factors, e.g., the time the mussels are able to isolate themselves from the toxic environment when exposed to a threshold concentration that triggers shell closure and the ensuing metabolic adjustments, and, on the other hand, the toxic effects elicited over time by the lower concentrations that did not cause shell closure for protection but an extended continuous exposure (which is the common scheme in exposure studies). Thus, it might be even unrealistic to expect a traditional dose/concentration-response curve since there are two overlapping mechanisms that take place under the different concentrations – and differences in the sensitivity of the individuals to the toxicant, not only in terms of toxicity as usual but in triggering shell closure in the same treatment.
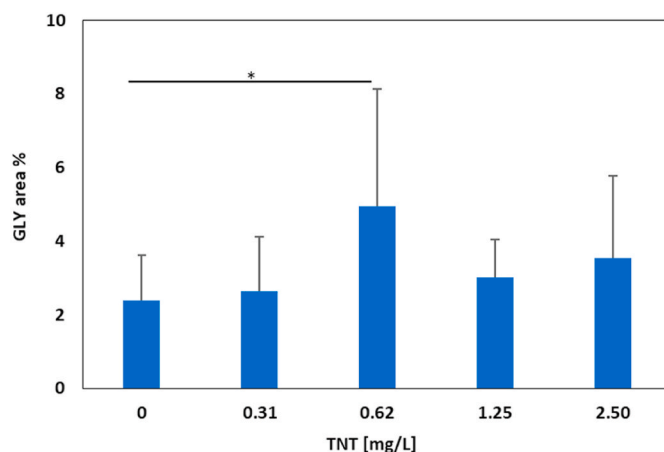
Fig. 13. Activities (mean +SD) of PK, PEPCK and ODH in the different exposure concentrations of dissolved TNT in the chronic exposure experiment. Significant differences to the control group (0) are given as * = p < 0.05, ** = p < 0.01 and *** = p < 0.001. Differences between the other treatment groups are given in Table A4.

Basing on ample previous research there is no doubt that TNT is a highly toxic substance for organisms. However, the biomarker results obtained in this study are not showing traditional dose/concentration-response curves and are therefore not easily interpreted. In most cases, significant changes in biomarkers measured in exposed mussels compared to the control group were detected. Nevertheless, taken the complexity of the biochemical/physiological system for the maintenance of homeostasis, some of the responses take opposite directions depending on the concentration range (e.g., the so-called bell-shape response in many enzymatic biomarkers). We follow the view that any disturbance from the normal (unexposed/control) is considered as an effect, which in case of such a well-known toxicant as TNT, is most probably a negative one (although the hormesis effect at low concentrations of toxic substances is often observed).
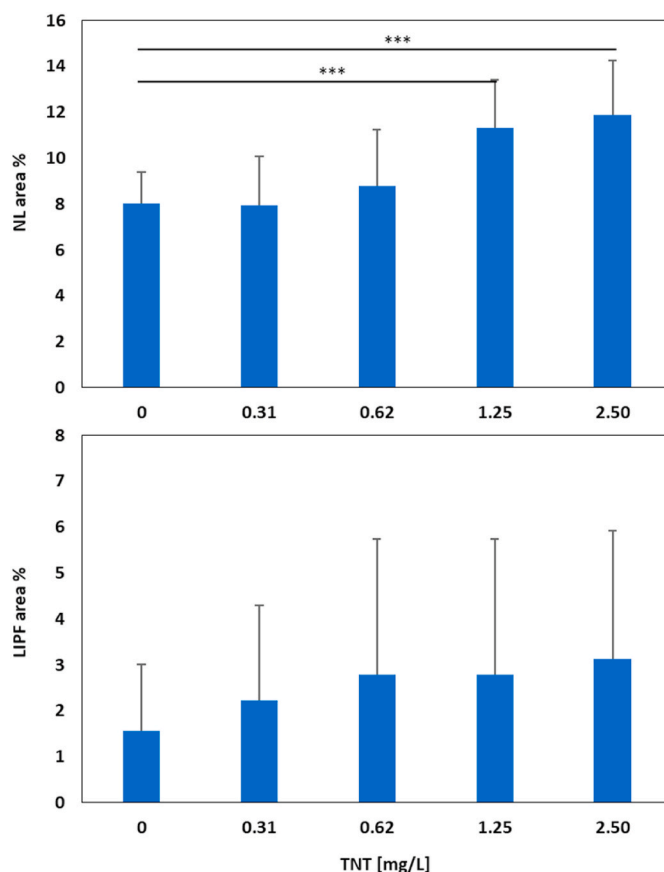

Fig. 14. Levels of NL and LIPF (area %, mean +SD) in the cells of the digestive gland tissue in the different exposure concentrations of dissolved TNT in the chronic toxicity test. Significant differences to the control group (0) are given as *** = p < 0.001. Differences between the other treatment groups are given in Table A4.

Fig. 15. Concentrations of GLY (area %, mean +SD) in the cells of the digestive gland in the different exposure concentrations of dissolved TNT in the chronic toxicity test. A significant difference to the control group (0) is given as * = p < 0.05. Differences between the other treatment groups are given in Table A4.

Acute toxicity is considered a somewhat different issue with mortality as an endpoint being the ultimate one and here observed only in the acute exposure experiment at 30 mg/L. The biomarker responses observed here represent negative, toxic effects at lower biological levels, and are indeed early-warning signals of possible more serious effects at higher levels, including a deteriorated health condition that reduces the
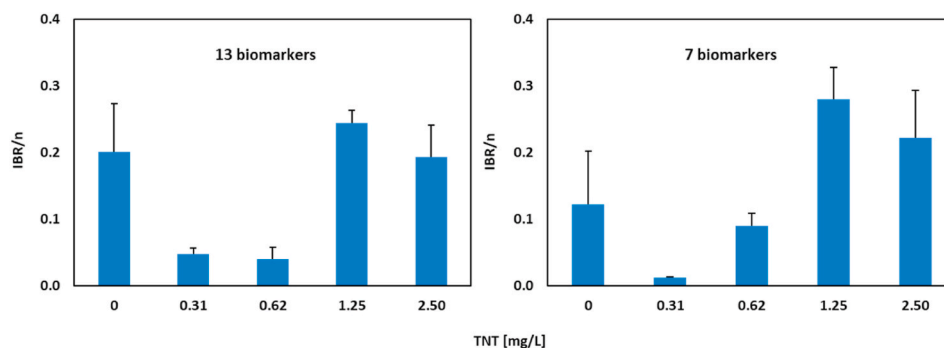
Fig. 16. Integrated Biomarker Response Index (IBR) divided by the number of biomarkers used in the calculation (IBR/n) in the different exposure concentrations in the chronic exposure experiment using 13 or 7 biomarkers for the calculation. Mean +SD (vertical bars), calculated from different data arrangements.

longevity of individuals at a longer time scale than the length of the experiment.

Application of the IBR index showed patterns that would easily remain undetected if looking only at single biomarkers, especially in cases where no apparent, statistically significant differences can be observed. However, subtle trends in different parameters are often present that together imply changes in the health status of organisms. In the acute toxicity experiment, the integrated response of mussels to the two lowest concentrations (0.31 and 0.62 mg/L) was clearly higher than that observed in the medium concentrations (1.25 and 2.50 mg/L). This strongly suggests that in the lower concentrations the mussels had not yet started to actively close their shells (and, subsequently, reduced their filtering activity, although not measured here) when detecting TNT in the water and became therefore more exposed to the chemical than those exposed to the intermediate concentrations. The IBR was also high at the two highest concentrations (5.0 and 10 mg/L), which is probably caused by the fact that increased shell closure was not sufficient to protect them from the toxic effects at this quite high concentration range.

Interestingly, the chronic exposure shows a quite different picture: the total stress level in the low exposure groups is even lower than in the control mussels while it is clearly higher at the intermediate concentrations. This suggests that during a 21 d chronic exposure, the effects of low levels of TNT (up to 0.62 mg/L) can be tolerated by mussels by physiological compensation mechanisms (which itself are energy-requiring) and these are activated by the low-level exposure. However, the original shell closure mechanism applied by mussels during the acute exposure period obviously cannot be maintained during a chronic exposure and thus does not function sufficiently as a protective measure anymore. As in the acute exposure, the mussels apparently can detect the presence of TNT at concentrations of 1.25 and 2.5 mg/L but need to open their shell more for the need for oxygen (and feeding under natural conditions), thus being exposed to the toxicant with subsequent biological effects since the physiological compensation mechanisms are insufficient to deal with the compound at this concentration range.

Finally, although in the chronic exposure no biomarker data is available concerning the high concentrations of 5.0 and 10 mg/L used in the acute exposure, the results of a few single biomarkers as well as the IBR assessments suggest that severe toxic effects would have occurred in mussels at these concentrations also under the chronic exposure.

5. Conclusions

The results obtained in this study showed the uptake of dissolved TNT by mussels according to the exposure concentrations and subsequent effects were measured using a battery of biomarkers, even at the lowest exposure concentrations used. Mussels seem to metabolize TNT to 4-ADNT and 2-ADNT, which are measured in their tissues together with the parent compound. Therefore, warfare materials remaining at the bottom of the seas pose an apparent and potential threat to marine ecosystems globally and further studies are urgently needed to assess this threat in detail. Finally, related to toxicity testing protocols in general, the TNT exposed mussels exhibited shell closure proportionally to the applied test concentrations, leading in some cases to unusual dose-response patterns in the biomarkers measured since it can significantly reduce the actual exposure to a substance. Such avoidance responses of organisms should be taken more carefully into account in toxicity assessments and in defining toxicity threshold values.

References

Appel, D., Strehse, J.S., Martin, H.-J., Maser, E., 2018. Bioaccumulation of 2,4,6-trinitrotoluene (TNT) and its metabolites leaking from corroded munition in transplanted blue mussels (*M. edulis*). Mar. Pollut. Bull. 135, 1072–1078.

Au, D.W.T., Wu, R.S.S., Zhou, B.S., Lam, P.K.S., 1999. Relationship between ultrastructural changes and EROD activities in liver of fish exposed to Benzo[a] pyrone. Environ. Pollut. 104 (2), 235–247.

Au, D.W.T., 2004. The application of histo-cytophatological biomarkers in marine polltution monitoring: a review. Mar. Pollut. Bull. 48, 817–834.

Ballentine, M., Tobias, C., Vlahos, P., Smith, R., Cooper, C., 2015. Bioconcentration of TNT and RDX in coastal marine biota. Arch. Environ. Contam. Toxicol. 68, 718–728.

Beck, A.J., van der Lee, E.M., Essert, A., Stamer, B., Gledhill, M., Schlosser, C., Achterberg, E.P., 2019. In situ measurement of explosive compound dissolution fluxes from exposed munition material in the Baltic Sea. Environ. Sci. Technol. 53 (10), 5652–5660.

Beddington, J., Kinloch, A.J., 2005. Munitions Dumped at Sea: A Literature Review. Imperial College London Consultants, London, UK, p. 90.

Bełdowski, J., Fabisiak, J., Popiel, S., Östin, A., Olsson, U., Vanninen, P., Lastumaki, A., Lang, T., Fricke, N., Brenner, M., Berglind, R., Baršiene, J., Klusek, Z., Pączek, B., Söderström, M., Lehtonen, K., Szubska, M., Malejevas, V., Koskela, H., Michalak, J., Turja, R., Bickmeyer, U., Broeg, K., Olejnik, A., Fidler, J., 2014. CHEMSEA findings. Gdańsk, Institute of Oceanology polish Academy of Sciences.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21 (6), 1316–1322.

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Brate, I.L.N., Schoyen, M., 2017. Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: a review. Mar. Environ. Res. 130, 338–365.

Bianchia, V.A., Langelohb, H., Urban, T., Krock, B., Müller, A., Bickmeyer, U., Abele, D., 2019. Separate and combined effects of neurotoxic and lytic compounds of Alexandrium strains on *Mytilus edulis* feeding activity and hemocyte function. Fish Shellfish Immunol. 84, 414–422.

Bickmeyer, U., Meinen, I., Meyer, S., Kröner, S., Brenner, M., 2020. Fluorescence measurements of the marine flatworm *Macrostomum lignano* during exposure to TNT and its derivatives 2-ADNT and 4-ADNT. Mar. Environ. Res. 161, 105041 submitted.

Bocquené, G., Galgani, F., 1998. Biological Effects of Contaminants: Cholinesterase Inhibition by Organophosphate and Carbamate Compounds. International Council for the Exploration of the Sea Copenhagen, Denmark.

Bolt, H.M., Degen, G.H., Doru, S.B., Plöttner, S., Harth, V., 2006. Genotoxicity and potential carcinogenicity of 2,4,6 – trinitrotoluene: Structural and toxicological considerations. Rev. Environ. Health 21 (4), 217–228.

Böttcher, C., Knobloch, T., Rühl, N., Sternheim, J., Wichert, U., Wöhler, J., 2011. Munitionsbelastung der deutschen Meeresgewässer – Bestandsaufnahme und Empfehlungen (Stand 2011). Bund/Länder Meeresprogramm für die Meeresumwelt von Nord- und Ostsee , ARGE BLMP Nord- und Ostsee, Sekretariat Bund/Länder-Messprogramm für die Meeresumwelt von Nord- und Ostsee (BLMP) im Bundesamt für Schifffahrt und Hydrographie, Hamburg, Germany, p. 172.

Bradford, M.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.

Brenner, M., Broeg, K., Frickenhaus, S., Buck, B.H., Köhler, A., 2014. Multi-biomarker approach using the blue mussel (*Mytilus edulis* L.) to assess the quality of marine environments: season and habitat related impacts. Mar. Environ. Res. 95, 13–27.

Brenner, M., Bostelmann, A., Klöpper, S., 2017. Military activities. In: Kloepper, S., et al. (Eds.), Wadden Sea Quality Status Report 2017. Common Wadden Sea Secretariat, Wilhelmshaven, Germany. Last updated 21.12.2017.

Broeg, K., Lehtonen, K.K., 2006. Indices for the assessment of environmental pollution of the Baltic Sea coasts: integrated assessment of a multi-biomarker approach. Mar. Pollut. Bull. 53, 508–522.

Claiborne, A., 1985. Handbook of Methods for Oxygen Radical Research. CRC Press, Boca Raton, Florida.

Culling, C.F.A., 1974. Handbook of Histopathological and Histochemical Techniques (Including Museum Techniques), third ed. Butterworth, London.

Farrington, J.W., Tripp, B.W., Tanabe, S., Subramanian, A., Sericano, J.L., Wade, T.L., Knap, A.H., 2016. Edward D. Goldberg proposal of the mussel watch: reflections after 40 years. Mar. Pollut. Bull. 110 (1), 501–510.

Fernández-Reiriz, M.J., Navarro, J.M., Contreras, A.M., Labarta, U., 2008. Trophic interactions between the toxic dinoflagellate *Alexandrium catenella* and *Mytilus chilensis*: feeding and digestive behaviour to long-term exposure. Aquat. Toxicol. 87, 245–251.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases - first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Johnson, L.R., Davenport, R., Balbach, H., Schaeffer, D.J., 1994. Near-ultraviolet light enhancement of microtox assays of trinitrotoluene and aminodinitrotoluenes. Ecotoxicol. Environ. Saf. 27, 23–33.

Jushasz, A.L., Naidu, R., 2007. Explosives: fate, dynamics and ecological impact in terrestrial and marine environments. Rev. Environ. Contam. Toxicol. 191, 163–215.

Koch, K., 2009. Subaquatische Kampfmittelaltlasten in der Ostsee – Neubewertung des Status Quo, Risikopotenzial und resultierende Handlungsszenarien, Thesis. Leuphania University Lüneburg, Germany, p. 393.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 113–390.

Lillie, R.D., Ashburn, L.L., 1943. Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxheimer's technique. Arch. Pathol. 36, 432–440.

Lima, D.R.S., Bezerra, M.L.S., Neves, E.B., Moreira, F.R., 2011. Impact of ammunition and military explosives on human health and the environment. Rev. Environ. Health 26 (2), 101–110.

Livingstone, D.R., Stickle, W.B., Kapper, M.A., Wang, S., Zurburg, W., 1990. Further studies on the phylogenetic distribution of pyruvate oxidoreductase activities. Comp. Biochem. Physiol. Part B: Comparative Biochemistry 97 (4), 661–666.

Livingstone, D.R., Pipe, R.K., 1992. Mussels and environmental contaminants: molecular and cellular aspects. In: Gosling, E.G. (Ed.), The Mussel Mytilus; Ecology, Physiology, Genetics, and Culture. Elsevier, Amsterdam, pp. 425–464.

Lotufo, G.R., Blackburn, W., Marlborough, S.J., Fleeger, J.W., 2010. Toxicity and bioaccumulation of TNT in marine fish in sediment exposures. Ecotoxicol. Environ. Saf. 73, 1720–1727.

Lotufo, G.R., 2011. Whole-body and body-part-specific bioconcentration of explosive compounds in sheepshead minnows. Exotoxicol. Environ. Saf. 74, 301–306.

Lotufo, G.R., 2012. Ecotoxicity of Explosives. Encyclopedia of aquatic ecotoxicology, pp. 328–336.

Lotufo, G.R., Rosen, G., Wild, W., Carton, G., 2013. Summary Review of the Aquatic Toxicology of Munitions Constituents. US Army Corps of Engineers, Engineers Research and Development Center, Environmental Laboratory, ERDC/EL TR-13-8, Vicksburg, US, p. 124.

Lotufo, G.R., Belden, J.B., Fisher, J.C., Chen, S.-F., Mowery, R.A., Chambliss, C.K., Rosen, G., 2016. Accumulation and depuration of trinitrotoluene and related extractable and nonextractable (bound) residues in marine fish and mussels. Environ. Pollut. 210, 129–136.

Lowe, D.M., Moore, M.N., Clarke, K.R., 1981. Effects of oil in the digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquat. Toxicol. 1, 213–226.

Mariussen, E., Stornes, S.M., Bøifot, K.O., Rosseland, B.O., Salbu, B., Heier, L.S., 2018. Uptake and effects of 2, 4, 6 - trinitrotoluene (TNT) in juvenile Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 194, 176–184.

Moore, M.N., Lowe, D.M., Koehler, A., 2004. Biological effects of contaminants: measurements of lysosomal membrane stability. In: ICES Techniques in Marine Environmental Sciences (TIMES), vol. 36. ICES, Copenhagen, p. 31.

Nipper, M., Carr, R.S., Biedenbach, J.M., Hooten, R.L., Miller, K., Saepoff, 2001. Development of marine toxicity data for ordnance compounds. Arch. Environ. Contam. Toxicol. 41, 308–318.

Nipper, M., Carr, R.S., Lotufo, G.R., 2009. Aquatic Toxicology of Explosives. Research Gate, vol. 4, pp. 77–115.

OECD, 2009. Guideline 230: OECD Guideline for the Testing of Chemicals; 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition.

OECD, 2014. Guideline 203: OECD Guideline for the Testing of Chemicals; Fish, Acute Toxicity Test.

Patterson, M.A., Parker, B.C., Neves, R.J., 1999. Glycogen concentration in the mantle tissue of freshwater mussels (Bivalvia: Unionidae) during starvation and controlled feeding. American Malacological Bulletin 15 (1), 47–50.

Pearse, A.G.E., 1985. Histochemistry: Theoretical and applied. In: Analytical Technology, fourth ed., vol. 2. Churchill Livingstone, Edinburgh, London, Melbourne and New York, p. 748.

Richter-Torres, P., Dorsey, A., Hodes, C., 1995. Toxicological Profile for 2,4,6-trinitrotoluene. U.S. Department of Health and Human Services, Public Health Service.

Rosen, G., Lotufo, G.R., 2007a. Bioaccumulation of explosive compounds in the marine mussel. Mytilus galloprovincialis. Ecotoxicol. Environ. Saf. 68, 237–245.

Rosen, G., Lotufo, G.R., 2007b. Toxicity of explosive compounds to the marine mussel *Mytilus galloprovinicialis* in aqueous exposures. Ecotoxicol. Environ. Saf. 68, 228–236.

Sabbioni, G., Rumler, R., 2007. Biomonitoring of workers cleaning up ammunition waste sites. Biomarkers 12 (6), 559–573.

Saka, M., 2004. Developmental toxicity of p,p'-Dichlorodiphenyltrichloroethane, 2,4,6-trinitrotoluene, their metabolites and benzo[a]pyrene in *Xenopus laevis* embryos. Environ. Toxicol. Chem. 23 (4), 1065–1073.

Salazar, M.H., Salazar, S.M., 1995. In-Situ bioassays using transplanted mussels: I. Estimating chemical exposure and bioeffects with bioaccumulation and growth. Environ. Toxicol. Risk Assess. 3, 216–241.

Strehse, J.S., Appel, D., Gerst, C., Martin, H., Maser, E., 2017. Biomonitoring of 2,4,6-Trinitrotoluene and degradation products in the marine environment with transplanted blue mussels (*Mytilus edulis*). Toxicology 380, 117–123.

Stuckas, H., Knöbel, L., Schade, H., Breusing, C., Hinrichsen, H., Bartel, M., Langguth, K., Melzner, F., 2017. Combining hydrodynamics modelling with genetics: can passive larval drift shape the genetic structure of Mytilus population? Mol. Ecol. 26 (10), 2765–2782.

Sun, Q., Bi, L., Su, X., Tsurugi, K., Mitsu, K., 2007. Valproate induces apoptosis by inducing accumulation of neutral lipids which was prevented by disruption of the *SIR2* gene in *Saccharomyces cerevisiae*. FEBS (Fed. Eur. Biochem. Soc.) Lett. 581, 3991–3995.

Talmage, S.S., Opresko, D.M., Maxwell, C.J., Welsh, C.J.E., Cretella, F., M, F., Reno, P.H., Daniel, F.B., 1999. Nitroaromatic munition compounds: environmental effects and screening values. In: Ware, G.W. (Ed.), Reviews of Environmental Contamination and Toxicology. Reviews of Environmental Contamination and Toxicology, vol. 161. Springer, New York, NY.

Viarengo, A., Canesi, L., 1991. Mussels as biological indicators of pollution. Elsevirs Aquaculture 94 (1–3), 225–243.

Viarengo, A., Nott, J.A., 1993. Mechanisms of heavy metal cation homeostasis in marine in- vertebrates. Comp. Biochem. Physiol. C Comp. Pharmacol. 104 (3), 355–372.

Vuori, K.A., Lehtonen, K.K., Kanerva, M., Peltonen, H., Nikinmaa, M., Berezina, N.A., Boikova, E., 2015. Oxidative stress biomarkers in the copepod *Limnocalanus macrurus*

from the northern Baltic Sea: effects of hydrographic factors and chemical contamination. Mar. Ecol. Prog. Ser. 538, 131–144.