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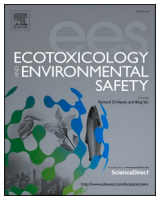

# Evaluating the toxicity of sea-dumped conventional and chemical munition degradation products to fish and human cells using a combination of cell viability assays

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ABSTRACT

The disposal of munitions in marine coastal areas after World Wars I and II has raised significant concerns about environmental contamination and human health risks. This study investigates the acute cytotoxicity of munition-related chemicals commonly detected near marine dumpsites, focusing on degradation products of explosives and related compounds (E&RC) and degradation products of chemical warfare agents and related compounds (CWA&RC). The research examines three CWA&RC (1,4-oxathiane, 1,4-dithiane, thiodiglycol) and four E&RC (2,4,6-trinitrotoluene, tetryl, 1,3-dinitrobenzene, picric acid) using the RTgill-W1 cell line (rainbow trout gill cells) as a proxy for fish toxicity and human cell lines (Caco2 and HepG2) to model potential human exposure via contaminated seafood. The results indicate low acute cytotoxicity of CWA&RC, while E&RC exhibit significantly higher toxicity. Notably, the EC10 and EC50 values for tetryl and 1,3-DNB in RTgill-W1 align with concentrations detected near North American dumpsites, reflecting environmentally relevant conditions. The study also reveals inter-species and inter-organ variability in toxicity mechanisms, identifying potential adverse outcome pathways such as AOP 220. These findings highlight the need for further research into chronic exposure scenarios at environmentally realistic concentrations and contribute crucial data to understanding the risks posed by the degradation products of these chemicals to aquatic life and human health.

Short Synopsis

Using fish and human cell lines, we assess toxicity of degradation products of explosives and chemical warfare agents at environmentally relevant concentrations in marine munition dumpsites. The study reveals significant risks for aquatic ecosystems and human health.

## 1. Introduction

The decades following World War I (WW I) and World War II (WW II) were marked by the intentional disposal of vast amounts of munition in the aquatic environment, with particular focus on the marine coastal areas of the country directly involved in the conflicts (Beck et al., 2018). Further, continuous exposure to marine conditions lead to the corrosion of the dumped munitions and consequent leakage and detection of munition compounds and their degradation products, such as explosives and related compounds (E&RC) and chemical warfare agents and related compounds (CWA&RC), in environmental samples collected in the vicinity of various dumpsites (Jurczak and Fabisiak, 2017; Koske et al., 2019). The detected concentrations, both in water and sediment samples, vary widely depending on the sampling location, yet usually ranging between tens and hundreds μg/L and μg/kg, respectively, as summarized by Barbosa et al. (2023). Additionally, various studies have reported the presence of E&RC (Appel et al., 2018; Beck et al., 2022; Koske, Goldenstein, et al., 2020; Koske, Straumer, et al., 2020; Strehse et al., 2017) and CWA&RC (Niemikoski et al., 2017, 2020) in marine biota, suggesting that humans may also be chronically exposed to such chemicals via the ingestion of contaminated seafood (Maser and Strehse,

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2020, 2021). Direct exposure to munitions is also possible, with OSPAR and the Helsinki Committee reporting approximately 900 accidental encounters per year, half of these happening due to entanglement in fishing nets (HELCOM, 2024; OSPAR, 2009).

Given the increasing human access to marine resources, i.e. food, space, and infrastructures, aligned with the recognition of the ocean as a new economic frontier, marine ecosystems face unprecedented pressures that increase the exposure to dumped munitions (Jouffray et al., 2020; Maser and Strehse, 2020, 2021). Consequently, the paucity of information on the impact of munition-related chemicals and their degradation products on human and environmental health, with special regards to CWA&RC, urges addressing.

Despite its historical background, limited information is available on the impact of these chemicals on human and environmental health. While previous studies addressed the acute toxicity of various E&RC to different aquatic species (Koske et al., 2019; Liang et al., 2017; Lotufo et al., 2010; Niemikoski et al., 2020; Nipper et al., 2001), similar studies with CWA&RC are limited to *Allivibrio fischeri* (Christensen et al., 2016) and *Daphnia magna* (Brzeziński et al., 2020; Czub et al., 2020a, 2020b). Recently, Wilczynski et al. (2023), (2024) studied of organoarsenic CWA in *Danio rerio* indicating significant chronic effects. This depicts the need to studied a broader set of CWAs for vertebrates particularly given the significant knowledge gaps. Nonetheless, robust information on potential acute and chronic impacts on aquatic organisms and humans is essential to be able to conduct environmental hazard and risk assessment of munition-related chemicals (ECHA, 2016).

One of the cornerstones of chemical environmental hazard and risk assessment is the quantification of acute toxicity towards fish, with regulatory agencies as the European Chemical Legislation REACH (Registration, Evaluation, Authorization and restriction CHemicals) and the US TSCA (Toxic Substances Control Act) requiring chemicals to be tested on fish according to an accepted test guideline, such as of the Organization for Economic Co-operation and Development (Fischer et al., 2019). However, the large number of animals required and high degree of severity imposed lead to the desire to develop *in vitro* alternatives that replace or supplement current animal procedures in accordance with the replacement, reduction, and refinement (3Rs) principle (de Wolf et al., 2007; Lillicrap et al., 2016). Also in human health hazard and risk assessment frameworks *in vitro* and *in silico* approaches have been prioritized over traditional *in vivo* testing, as the former have proven to be reliable and relevant, capable of mimicking human biology and providing mechanistic information about how a chemical may cause toxicity in humans (Ingenbleek et al., 2021; Stucki et al., 2022; van der Zalm et al., 2022). In line with the ongoing demand for alternative approaches, a new methodology was established for the assessment of fish acute toxicity using the permanent fish gill cell line from rainbow trout (*Oncorhynchus mykiss*), RTgill-W1 (Bols et al., 1994; Fischer et al., 2019; OECD, 2021) The now-validated OECD test guideline assesses the impact on cell viability via the combination of three fluorometric assays, ultimately allowing an *in vitro* to *in vivo* extrapolation of data that can be used in risk assessment strategies (OECD, 2021). Following the same rationale, the described array of assays was used to assess the impact of a variety of xenobiotics on human health (Rudzok et al., 2009; Ude et al., 2017).

In this study, the toxicity of three CWA&RC, i.e. 1,4-oxathiane, 1,4-dithiane, and thiodiglycol, and four E&RC, i.e. 2,4,6-trinitrotoluene (TNT), tetryl, 1,3-dinitrobenzene (1,3-DNB) and picric acid, was assessed. Specifically, fish acute toxicity was assessed using the RTgill-W1 cell line as a proxy, as described in OECD guideline 249 (OECD, 2021). Further, given the aforementioned potential for human exposure via contaminated seafood, the impact on the human intestine and liver was assessed using the human colon adenocarcinoma (Caco2) and human hepatocellular carcinoma (HepG2) cell lines as respective proxies. The gathered information on seven commonly detected munition-related products and degradation products provides a first insight into the acute cytotoxicity of these chemicals in fish and humans, hence contributing to understanding the impact of these chemicals on human and environmental health.

## 2. Materials and methods

### 2.1. Test chemicals, stock, and dosing solutions

Seven munition-related chemicals frequently detected in munition dumpsites were tested, of which three sulfur mustard related chemicals, 1,4-oxathiane, 1,4-dithiane, and thiodiglycol, and two explosives and related chemicals, TNT, tetryl, 1,3-DNB, and picric acid. A list of the tested chemicals and corresponding relevant physio-chemical properties, namely the molecular weight and octanol-water partition coefficient (log Kow), is provided in Table 1. Stock solutions of 1,4-oxathiane, 1,4-dithiane, thiodiglycol, and picric acid were prepared in ultra-pure water while TNT, tetryl, and 1,3-DNB stock solutions were prepared in methanol. The stock solutions of all seven chemicals were prepared and provided by the Belgian Department of Defense (DLD) and the Royal Maritime Academy (RMA).

Exposure solutions were prepared as described in OECD 249 (OECD, 2021). Exposure solutions of 1,4-oxathiane, 1,4-dithiane, thiodiglycol, and picric acid were prepared via serial dilution in L-15/ex (1:2). On the other hand, TNT, tetryl, and 1,3-DNB stock solutions were first serially diluted in methanol (1:2). Each of the resulting solutions was subsequently diluted in the respective exposure media (at a ratio of 1:200), i.e. L-15/ex and DMEM, to achieve the final dosing solutions for tests with RTgill-W1, and Caco2 and HepG2, correspondingly. Exposure concentrations ranges were defined based on predicted ecotoxicity data retrieved from ECOSAR v2.2 (https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-model). A maximum nominal concentration was established at 100 mg/L ensuring the exclusion of completely unrealistic concentrations while complying with internal safety measures.

### 2.2. Routine cell culture

Routine cell culture of rainbow trout (*Oncorhynchus mykiss*) gill cell

**Table 1**
Physicochemical properties of the tested chemical warfare agents and related chemicals and explosives and related chemicals.

| Chemical name or abbreviation | CASNR | Class | Molecular Weight (g/mol)[a] | Octanol-water partition coefficient (log Kow)[a] |
|---|---|---|---|---|
| 1,4-oxathiane | 15980–15–1 | Sulfur mustard related chemical | 104.17 | 0.742 |
| 1,4-dithiane | 505–29–3 | Sulfur mustard related chemicals | 120.23 | 0.77 |
| Thiodiglycol | 111–48–8 | Sulfur mustard related chemicals | 122.19 | −0.630 |
| TNT | 118–96–7 | Parent compound | 227.13 | 1.6 |
| Tetryl | 479–45–8 | Parent compound | 287.17 | 1.69 |
| Picric acid | 88–89–1 | Tetryl related chemical | 229.10 | 1.33 |
| 1,3-DNB | 99–65–0 | TNT related chemical | 168.12 | 1.55 |

[a] - Data retrieved from US EPA CompTox Chemical Dashboard (https://comptox.epa.gov/dashboard/), PubChem (https://pubchem.ncbi.nlm.nih.gov/).

line RTgill-W1 (ATCC® CRL-2523™, Manassas, Virginia) (Bols et al., 1994) was performed at 19°C ± 1°C in 75 $cm^2$ cell culture flasks (VWR, 734–2313, Haasrode, Belgium) with L-15 culture medium without phenol red (Gibco™, 21083027, Gosselies, Belgium) supplemented with 5 % fetal bovine serum (FBS) (Sigma Aldrich, F7524, Hoeilaart, Belgium) and 1 % gentamicin sulfate solution (10 mg/mL) (Gibco™, 15710049, Gosselies, Belgium).

Similarly, the human colon adenocarcinoma cell line Caco2 (ATCC® HTB-37™, Manassas, Virginia) and the human hepatocellular carcinoma cell line HepG2 (ATCC® HB-8065™, Manassas, Virginia) were routinely kept in culture at 37°C in a humidified 5 % $CO_2$-95 % atmosphere in 75 $cm^2$ cell culture flasks (VWR, 734–2313, Haasrode, Belgium) with DMEM medium (ThermoFisher, 61965–026, Gosselies, Belgium) supplemented with 10 % heat-inactivated FBS (ThermoFisher, 10082–147, Gosselies, Belgium) and 1 % penicillin-streptomycin solution (5000 U/mL) (ThermoFisher, 15070–063, Gosselies, Belgium).

### 2.3. RTgill-W1 acute toxicity assays

The cytotoxicity of the seven selected munition-related chemicals in RTgill-W1 was assessed in 24-well plates (VWR, 734–2325, Haasrode, Belgium) following the guidelines described in OECD 249 (OECD, 2021).

RTgill-W1 cells were seeded in 22 out of the 24-wells at 350 000 cells/mL of cell culture media. Given the formation of a confluent monolayer within 24–48 hours (h), confirmed by microscopy, chemical exposure was initiated.

Exposure of cells to the chemicals was carried out with the previously described dosing solutions in L-15/ex (cfr. *Test chemicals, stock, and dosing solutions*). L-15/ex is a simplified version of the L-15 cell culture media containing only the salts, galactose, and pyruvate of the complete L-15 medium (Schirmer et al., 1997). Exposure to the test chemicals was initiated by rinsing the RTgill-W1 confluent monolayer once with 1 mL of L-15/ex. Subsequently, 2.5 mL of the respective dosing solution were transferred to the assigned wells, with each concentration being dosed in triplicates. Additionally, the solvent control (methanol) was dosed in triplicates when applicable (tests with TNT, tetryl, and 1,3-DNB). The two wells without cells were used as controls to detect background fluorescence by the test chemicals as this might interfere with the fluorescence of the cell viability indicator dyes (Schirmer et al., 2000). Specifically, one of the cell-free control wells receives L-15/ex exposure medium only whereas the other receives L-15/ex exposure medium with the highest chemical concentration (in methanol when applicable). After dosing, 500 µL aliquots were collected from each well and placed in previously labeled amber vials for chemical analysis ($C_{0\,h}$). Further, the well-plates were sealed with an adhesive foil and placed in the incubator at 19°C ± 1°C. After 24 h, exposure was terminated by removing the adhesive foil and collecting a 500 µL aliquot from each vial for chemical analysis ($C_{24\,h}$).

Cell viability was then measured using a combination of three fluorometric assays, alamarBlue™ (AB) (Invitrogen, DAL1100, Zaventem, Belgium) to measure metabolic activity, Neutral Red (NR) (Sigma-Aldrich, N2889, Hoeilaart, Belgium) to measure lysosomal membrane integrity, and CFDA-AM (Invitrogen, C1345, Zaventem, Belgium) to measure cell membrane integrity. Fluorescence was measured in a Tecan Infinite 200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) with the excitation/emission wavelength suggested in OECD 249, i.e. excitation: 530 nm and emission: 595 nm for AB, excitation: 530 nm and emission: 645 nm for NR, excitation: 493 nm and emission: 541 nm for CFDA-AM.

### 2.4. Caco2 and HepG2 acute toxicity assays

The aforementioned procedure was also followed to assess the effects of the selected chemicals in the human cell lines, Caco2 and HepG2, albeit with a few modifications. Specifically, cells were seeded at 120,000 cells/ mL of culture media in 22 of the 24 wells, having two independent plates per tested chemical hence allowing the assessment of cell viability after 24 h and 48 h of exposure.

After 24 h, exposure was initiated by transferring 2.5 mL of the previously prepared dosing solutions (cfr. *Test chemicals, stock, and dosing solutions*) to the corresponding wells, in triplicates, and immediately collecting 500 µL aliquots into amber vials for chemical analysis ($C_{0\,h}$). Also here, the two cell-free wells were used as controls to detect background fluorescence. The well-plates sealed with an adhesive foil were then placed in the incubator at 37°C in a humidified 5 % $CO_2$-95 % atmosphere. Exposure was terminated and 500 µL aliquots were collected into labeled amber vials for chemical analysis after 24 h ($C_{24\,h}$) and 48 h ($C_{48\,h}$).

The same combination of assays, i.e. AB, NR, and CFDA-AM, was used to assess cell viability under the previously described conditions.

### 2.5. Data analysis

The raw fluorescence readings from each cell viability assay were recorded and expressed as "percentage to the control" in order to calculate cell viability per treatment, compared to the respective control. Concentrations leading to 10 % ($EC_{10}$), and 50 % ($EC_{50}$) reduction in cell viability were determined by non-linear regression sigmoidal dose-response curve fitting using the R drm package's drc function (R Development Core Team, 2022). Further, the no-observed effect concentrations (NOEC) were derived via one-way ANOVA followed by a Tukey's post-hoc HSD test, using the R aov (analysis of variance) and TukeyHSD functions, respectively (R Development Core Team, 2022). Chemical concentrations were considered as the geometric mean of the concentrations at the beginning ($C_{0\,h}$) and at the end of exposure ($C_{24\,h}$/$C_{48\,h}$), as suggested in OECD guideline 249 (OECD, 2021). NOEC, $EC_{10}$, and $EC_{50}$ values were estimated based on the determined chemical concentrations, and the lowest used as the most conservative (eco) toxicity estimate.

### 2.6. Chemical extraction and quantification

Chemical analysis was performed on the 500 µL aliquot samples collected from the wells at the beginning ($C_{0\,h}$) and end of exposure ($C_{24\,h}$/$C_{48\,h}$) that were immediately stored at −20°C. Specifically, two replicates of the highest tested concentrations were analyzed per tested cell line, per chemical, and per time point (Table S1, Table S2 and Table S3). Intermediate concentrations were estimated based on the used dilution factor (1:2). The chemical concentrations in the samples was determined by gas chromatography–mass spectrometry (GC-MS) for 1,4-oxathiane and 1,4-dithiane, liquid chromatography–mass spectrometry (LC–MS) for thiodiglycol and picric acid, and GC-MS/MS for 2,4,6-TNT, tetryl and 1,3-DNB. The respective limits of detection and quantification can be found in Table S5. Calibration curves per chemical can be found in Figs. S1 to S7.

Tetryl, 2,4,6-TNT and 1,3-DNB were analyzed by gas chromatography coupled to a triple quadrupole mass spectrometer (GC-TSQ, Thermo Scientific, Bremen, Germany) at RBINS, OD Nature, ECOCHEM. The 500 µL aliquot samples were diluted to 4 mL, and 1–4 mL of the samples (depending on the concentration) were extracted with dichloromethane (2 × 2 mL) and subsequently concentrated to 1 mL under a gentle $N_2$ stream. 1,3-DNB-D4 and 2,4,6-TNT-$C_{13}N_{15}$ were added as internal standard. A recovery standard was added just before the samples were brought on the GC. One µL of the extract was injected in splitless mode into the GC system. The pressure value for high-pressure injection was 250 kPa, and the injection port temperature was set at 40 °C. The chromatographic separation was performed on a RXI-5sil MS column (20 m length, 0.15 mm internal diameter, 0.18 µm film thickness, Restek, Bad, Homburg, Germany) with Helium alphagas 2 (constant flow 1 mL/min) as the carrier gas. The temperature program of the column was set to maintain the initial temperature at 35 °C for

2 min, then increasing the temperature to 250 °C at a rate of 25 °C per minute, further increasing the temperature to 300 °C at a rate of 10 °C per minute, and finally holding for 4.5 min at 300 °C. To determine the concentrations of 1,4-oxathiane and 1,4-dithiane 200 the sample (200 μL) was extracted with dichloromethane (2 ×250 μL). The extract was made up to 1 mL with dichloromethane and analysed with gas chromatography coupled to a single quadrupole mass spectrometer (Agilent 7890B series GC System + Agilent 5977B MSD, Diegem, Belgium). To determine the concentrations of thiodiglycol and picric acid the samples were diluted with ultrapure water in in such a way that the measurement results fell in the range from 0 to 1000 μg/L. The diluted samples were analyzed by liquid chromatography coupled to a high resolution mass spectrometer (Bruker Elute UHPLC + Bruker Compact II, Kontich, Belgium).

## 3. Results

The impact of the seven selected munition-related chemicals on cell viability was assessed. The obtained results are discussed in the following sub-sections, discriminating between fish, specifically the RTgill-W1 cell line, and human cell lines, namely Caco2 and HepG2. Toxicity data based on analytical concentrations are provided for all chemicals, except for tetryl, following assays with Caco2 and HepG2. For these, analytical data could not be retrieved due to difficulties in chemical extraction and quantification. This was attributed to the used exposure media, i.e. DMEM medium (ThermoFisher, 61965–026, Gosselies, Belgium) supplemented with 10 % heat-inactivated FBS (ThermoFisher, 10082–147, Gosselies, Belgium) and 1 % penicillin-streptomycin solution (5000 U/mL) (ThermoFisher, 15070–063, Gosselies, Belgium), which highly interferes with chemical analysis and was confirmed in separate experiments (Table S4). Nonetheless, tetryl could be analyzed in the stock solutions in pure methanol. Table 2 summarizes the generated toxicity data following 24 h of exposure, for RTgill-W1, and 24 h and 48 h of exposure for Caco2 and HepG2.

Concentration-response curves were obtained for the seven tested chemicals for three response variables, i.e. metabolic activity, cell membrane integrity, and lysosomal integrity as measures of cell viability (see Fig. 1 and Fig. 2). The combination of the three aforementioned fluorometric assays allowed the estimation of the NOEC, $EC_{10}$, and $EC_{50}$ values for the seven tested munition-related chemicals. These values were determined based on the most sensitive assay, ensuring a conservative estimate in accordance with the guidelines outlined in the OECD test guideline 249 (OECD, 2021).(Fig. 3).

Within the tested CWA&RC, 1,4-dithiane presented the highest

**Table 2**
Summary of the estimated (eco)toxicity values resulting from the concentration-response curves of the tested CWA&RC and E&RC for RTgill-W1, Caco2, and HepG2. The NOEC, $EC_{10}$, and $EC_{50}$ values, and corresponding 95 % confidence intervals between brackets, are presented in mg/L for the seven chemicals tested in RTgill-W1, Caco2, and HepG2 cell lines. These values represent the (eco)toxicity data obtained from the most sensitive cell viability assay among the three tested. Please note that the NOEC represents an observed concentration, hence dependent on the tested concentration. The $EC_{10}$, on the other hand, was estimated based on an interpolation within a non-linear sigmoidal curve resulting from the complete concentration range.

| | | RTgill-W1 | Caco2 | | HepG2 | |
|---|---|---|---|---|---|---|
| | | 24 h | 24 h | 48 h | 24 h | 48 h |
| **CWA&RC (all values in mg/L)** | | | | | | |
| Thiodiglycol | NOEC | 60.12 | 56.44 | 52.42 | 11.67 | ND* |
| | $EC_{10}$ | 125.03 (89.86 – 173.96) | 56.50 (30.49 – 104.69) | 51.82 (30.59 – 87.80) | ND | ND |
| | $EC_{50}$ | ND | ND | 118.46 (95.44 – 147.02) | ND | ND |
| 1,4-oxathiane | NOEC | 41.02 | 45.57 | 44.67 | 7.35 | 38.89 |
| | $EC_{10}$ | 78.84 (66.40 – 93.85) | 54.53 (38.12 – 78.00) | 28.16 (13.52 – 58.66) | ND | ND |
| | $EC_{50}$ | ND | ND | ND | ND | ND |
| 1,4-dithiane | NOEC | 54.51 | 43.89 | 32.31 | 23.85 | 40.07 |
| | $EC_{10}$ | 24.23 (12.32 – 47.64) | 60.75 (38.46 – 95.96) | 47.85 (32.45 – 70.55) | 33.78 (20.47 – 55.74) | 56.80 (44.46 – 72.57) |
| | $EC_{50}$ | ND | ND | ND | 76.74 (68.32 – 86.20) | 82.79 (80.03 – 85.64) |
| **E&RC (all values in mg/L)** | | | | | | |
| TNT | NOEC | ND* | ND* | 2.18 | 6.51 | 6.00 |
| | $EC_{10}$ | 9.73 (5.68 – 16.66) | 3.36 (2.74 – 4.13) | 3.97 (2.84 – 5.57) | 8.01 (6.56 – 9.78) | 12.10 (10.11 – 14.47) |
| | $EC_{50}$ | 23.23 (18.99 – 28.41) | 9.21 (8.37 – 10.14) | 8.03 (7.44 – 8.66) | 28.47 (26.30 – 30.83) | 20.84 (18.94 – 22.94) |
| Tetryl | NOEC | ND* | 6.25# | 6.25# | 6.25# | 12.50# |
| | $EC_{10}$ | 0.19 (0.03 – 1.38) | 6.85# (4.92 – 9.55) | 10.77# (5.12 – 22.68) | 5.83# (4.99 – 6.81) | 23.95# (21.51 – 26.66) |
| | $EC_{50}$ | 0.58 (0.21 – 1.63) | 16.93# (15.37 – 18.64) | 50.87# (37.43 – 69.14) | 14.37# (13.42 – 15.40) | 39.45# (37.59 – 41.41) |
| 1,3-DNB | NOEC | ND* | ND* | 1.65 | 13.01 | 11.57 |
| | $EC_{10}$ | 0.18 (0.03 – 1.03) | 2.04 (1.05 – 3.98) | 1.80 (1.09 – 2.97) | 16.54 (10.28 – 26.58) | 10.92 (4.17 – 28.07) |
| | $EC_{50}$ | 18.90 (11.27 – 31.67) | 16.77 (12.58 – 22.35) | 8.12 (6.31 – 10.46) | ND | 49.05 (42.93 – 56.04) |
| Picric acid | NOEC | ND* | 62.63 | 62.02 | 31.29 | 15.57 |
| | $EC_{10}$ | 28.18 (15.25 – 52.06) | 50.72 (29.34 – 87.69) | 30.79 (18.02 – 52.62) | 21.01 (10.58 – 41.72) | 28.78 (20.54 – 40.31) |
| | $EC_{50}$ | 65.44 (53.64 – 79.83) | ND | 148.26 (114.31 – 192.30) | ND | 168.97 (141.93 – 201.16) |

ND – not determined.
# – nominal concentrations due to impossible chemical quantification.
*– significant effects were observed at the lowest tested concentration.
*RTgill-W1 acute* toxicity *based on measured exposure concentrations*

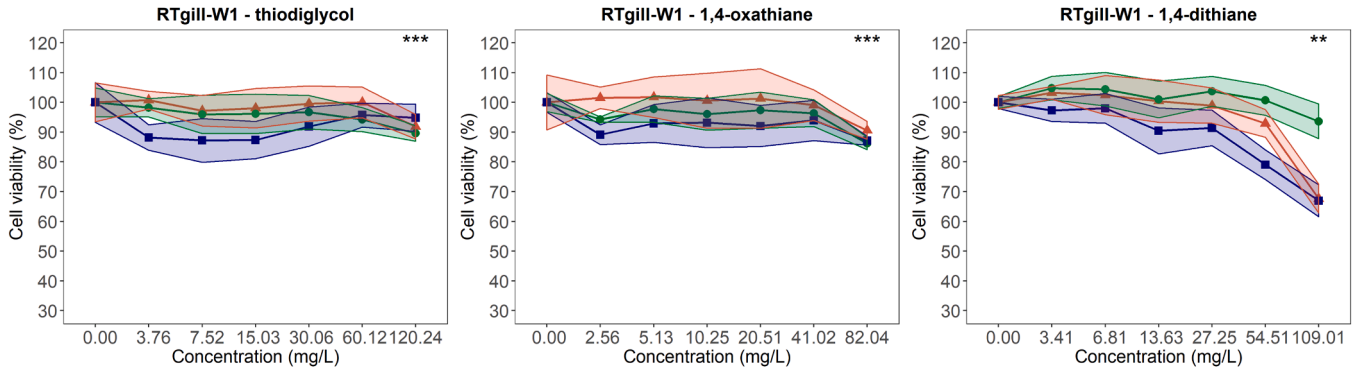


**Fig. 1.** Concentration-response curves of the tested CWA&RC for the three cell viability measures for RTgill-W1, following 24 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. * indicates statistically significant difference in growth rate when compared to the control treatment (Tukey's post-hoc HSD test, * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

cytotoxicity to RTgill-W1, followed by 1,4-oxathiane and finally thiodiglycol. Due to the low toxicity caused by the chemicals, no $EC_{50}$ values could be estimated. Nonetheless, NOEC and $EC_{10}$ were estimated, showing 1,4-dithiane to be the most toxic of the CWA&RC with an estimated EC10 of 24.23 mg/L (see Table 2). There, the NOEC reported for 1,4-dithiane is lower than the estimated $EC_{10}$. While these values tend to be in the same range, the NOEC represents an observed concentration, hence dependent on the tested concentration. The $EC_{10}$, on the other hand, was estimated based on an interpolation within a non-linear sigmoidal curve resulting from the complete concentration range. Further, none of the three assays showed particular sensitivity to thiodiglycol, 1,4-oxathiane, and 1,4-dithiane.

The tested E&RC, on the other hand, present much higher toxicity, leading to 100 % cell mortality at some of the tested concentrations (see Fig. 2). Tetryl caused the highest cytotoxicity, followed by 1,3-DNB, TNT, and picric acid (see Table 2). Importantly, according to OECD 249, the estimated $EC_{50}$ *in vitro* values, i.e. 0.58 mg/L for tetryl, 18.90 mg/L for 1,3-DNB, 23.23 for TNT, and 65.44 for picric acid, corresponding to the predicted 96 h lethal concentration to 50 % of the exposed organisms (LC50) in fish (OECD, 2021). Noteworthy is the fact that TNT, 1,3-DNB, and picric acid particularly impact the cells' metabolic activity, here assessed via the AB assay, well before affecting the other two assays hence potentially providing insight into the mode of action of these chemicals (see Fig. 2, AB assay represented in blue).

### 3.1. *Caco2 and HepG2 acute toxicity based on measured exposure concentrations*

As for RTgill-W1, concentration-response curves were derived from the three used cell viability assays for the seven tested munition-related chemicals following 24 h and 48 h of exposure.

The estimated cytotoxicity data for Caco2 suggested that none of the three tested CWA&RC have a high impact on cell viability. Given the low cytotoxicity, only for thiodiglycol could the $EC_{50}$ value following 48 h of

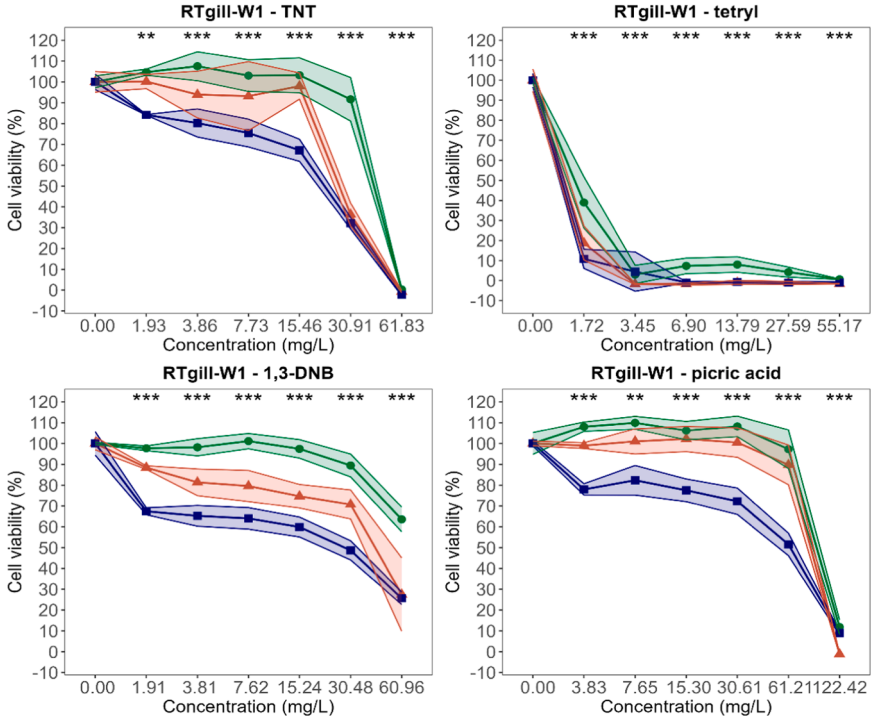


**Fig. 2.** Concentration-response curves of the tested E&RC for the three cell viability measures for RTgill-W1, following 24 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. * indicates statistically significant difference in growth rate when compared to the control treatment (Tukey's post-hoc HSD test, * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

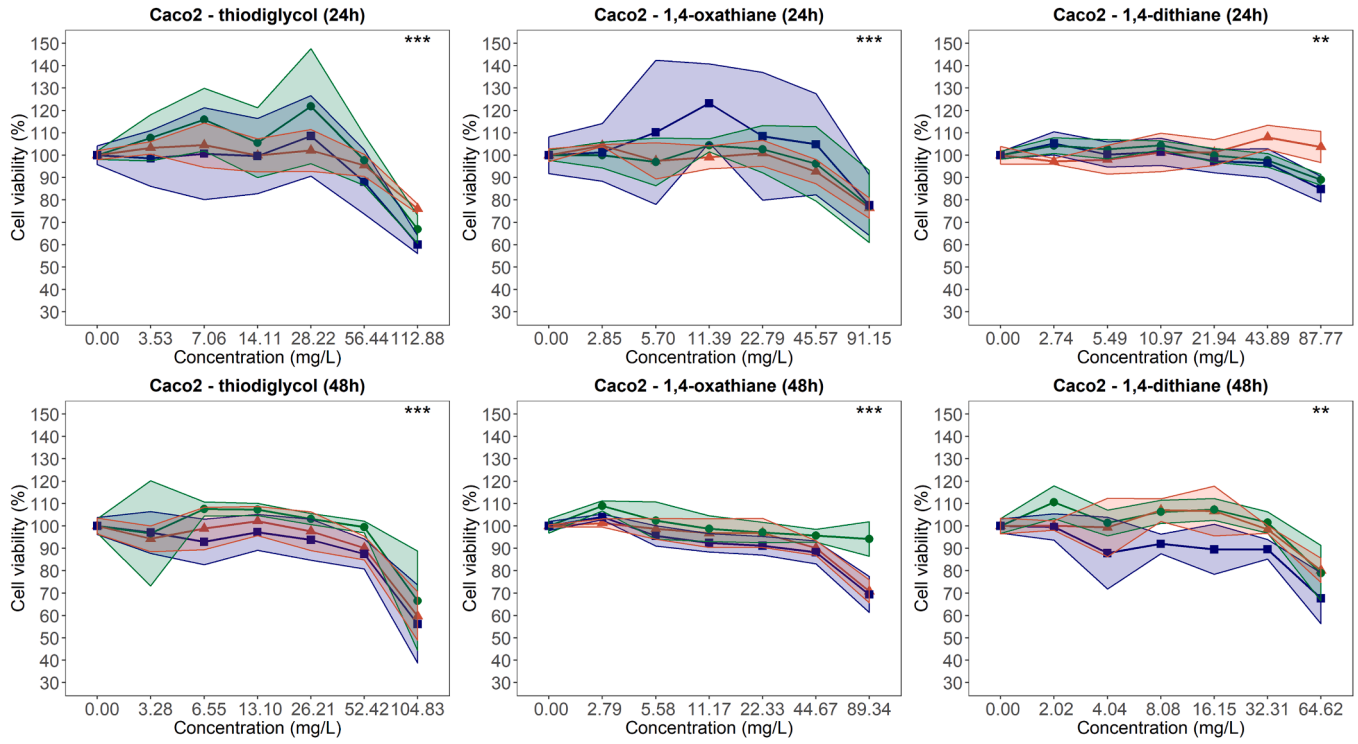


**Fig. 3.** Concentration-response curves of the tested CWA&RC for the three cell viability measures for Caco2, following 24 h and 48 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. * indicates statistically significant difference in growth rate when compared to the control treatment (Tukey's post-hoc HSD test, * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

exposure be estimated ($EC_{50\text{-}24h} = 118.46$ mg/L). NOEC and $EC_{10}$ values were estimated for the three tested chemicals following 24 h and 48 h of exposure with the results showing slightly higher toxicity for both 1,4-oxathiane and 1,4-dithiane after 48 h of exposure, when compared to the data following 24 h of exposure (see Table 2). Moreover, none of the assays was particularly sensitive to the tested CWA&RC.

Also for Caco2 the E&RC are more toxic than the CWA&RC. Here, NOEC, $EC_{10}$, and $EC_{50}$ values were estimated for all four tested chemicals, except for the picric acid $EC_{50\text{-}24h}$. Results show that, when considering the estimated $EC_{50}$ values, TNT poses the highest toxicity, followed by 1,3-DNB, tetryl, and picric acid (see Table 2). Additionally, a comparison between the estimated toxicity values for both exposure periods shows that prolonged exposure to TNT, 1,3-DNB, and picric acid lead to high cytotoxicity, corroborated by the chemicals' stability in the exposure media for the duration of the assays (Table S2). On the other hand, tetryl, for which nominal data is used due to the impossible quantification of the chemical in the exposure media of Caco2 and HepG2, appears to degrade or bind to either media components, such as FBS, or the well plate itself, within the duration of the assays, only interacting with the cell during the earlier hours of exposure (Table S4). Further, the higher sensitivity of the NR assay to TNT, 1,3-DNB, and picric acid suggests that these chemicals interact with Caco2 by specifically targeting the cell membrane (see Fig. 4, NR assay represented in red).

Interestingly, cytotoxicity data for HepG2 suggest that this cell line is the least sensitive to thiodiglycol and 1,4-oxathiane and the most sensitive to 1,4-dithiane. $EC_{10}$ and $EC_{50}$ values could be estimated for 1,4-dithiane (see Table 2). Furthermore, contrarily to thiodiglycol and 1,4-oxathiane, 1,4-dithiane appears to more severely affect the integrity of the cell membrane (see Fig. 5, NR assay represented in red).

In general, the least sensitive of the tested cell lines to E&RC is HepG2. The estimated toxicity values show that TNT most severely impacts HepG2 cell viability while 1,3-DNB only affects the cells at concentrations higher than those observed for Caco2. The comparison of chemical sensitivity between the two exposure periods shows that HepG2 follows the same trend as Caco2, i.e. increased effects with prolonged exposure to E&RC, except for tetryl (see Table 2). Interestingly, when exposed to HepG2 none of the chemicals seem to specifically target any of the assays, in opposition to the observed sensitivity of NR following exposure of Caco2 to TNT, 1,3-DNB, and picric acid (see Fig. 6). Such results suggest that the chemicals' mode of action may not only be species-specific (AB was targeted in the assays with RTgill-W1) but also organ-specific in humans.

## 4. Discussion

The tested degradation products of CWA&RC, i.e. thiodiglycol, 1,4-oxathiane, and 1,4-dithiane, show low cytotoxic potential to the fish cell line RTgill-W1. Specifically, despite the absence of $EC_{50}$ data, the $EC_{10}$ values suggest that 1,4-dithiane is the most toxic to fish. The estimated $EC_{10}$ values provide important insight into the early effects of these chemicals on fish health and are indicative of thresholds for chronic toxicity (Beasley et al., 2015). The $EC_{10}$ value here estimated for 1,4-dithiane ($EC_{10} = 24.23$ mg/L) is well in line with the fish chronic value predicted by ECOSAR (ChV = 32.25 mg/L) (manuscript in preparation). Even though the toxicity values are well above environmental concentrations, usually reported in the range of tens to hundreds of μg/L (Bełdowski et al., 2016; Bolt et al., 2006; Koske et al., 2019), the fact that these assays target cell viability suggests that other processes are likely to be triggered much earlier, and at much lower concentrations, in the cascade of events leading to the observed outcome (Fay et al., 2017). This is particularly concerning given the ubiquitous presence of 1,4 dithiane in Baltic sediments from the chemical munition dumpsites and the fact that thiodiglycol, most commonly studied degradation compound of the same parent compound, was generally considered a low toxicity compound, (Vanninen et al., 2020). With regards to the tested E&RC, the estimated *in vitro* $EC_{50}$, which corresponds to the *in vivo* 96 h $LC_{50}$, show relatively good alignment with those previously reported for

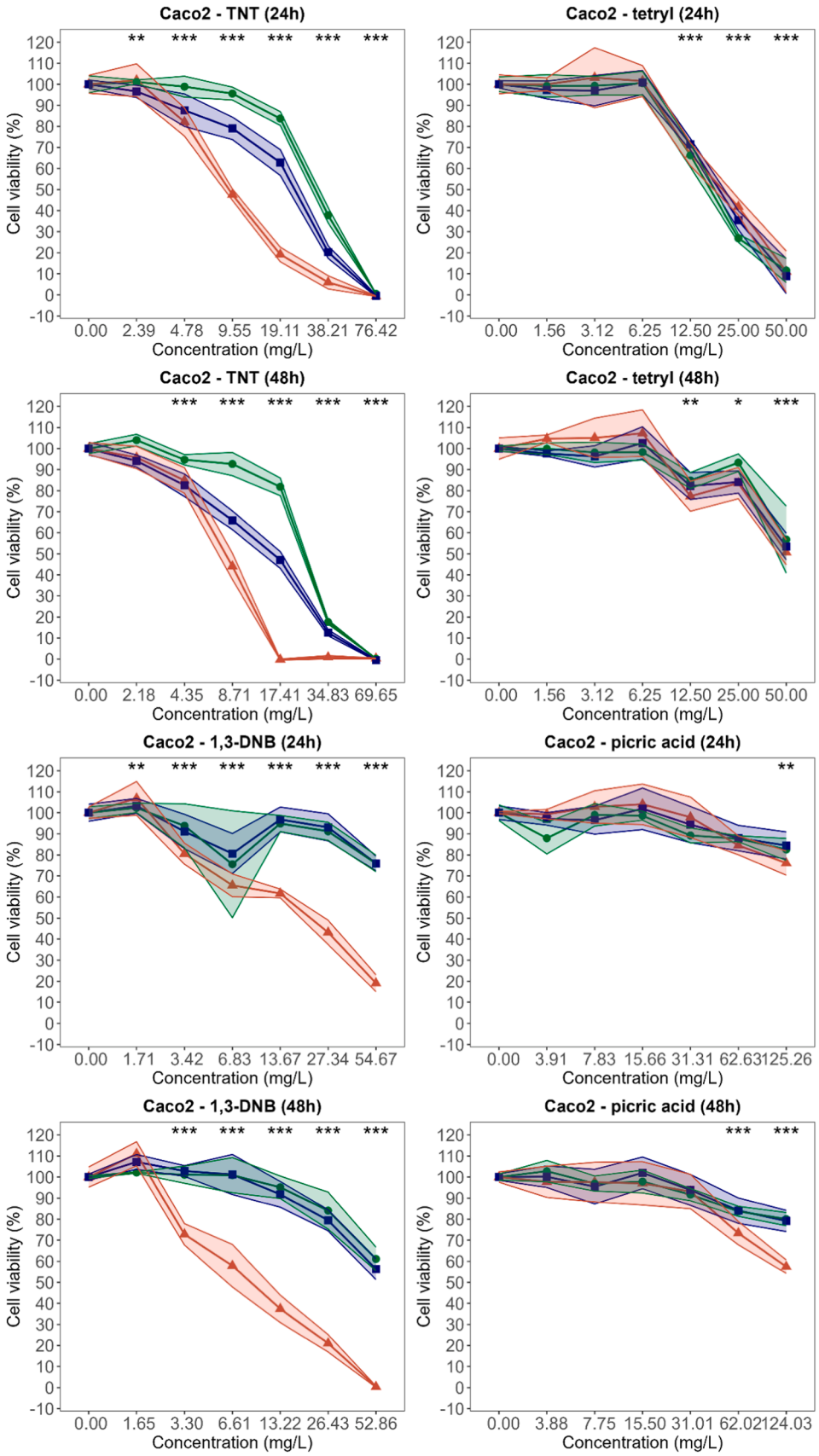


**Fig. 4.** Concentration-response curves of the tested E&RC for the three cell viability measures for Caco2, following 24 h and 48 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. * indicates statistically significant difference in growth rate when compared to the control treatment (Tukey's post-hoc HSD test, * - $p < 0.05$, ** - $p < 0.01$, and *** - $p < 0.001$.

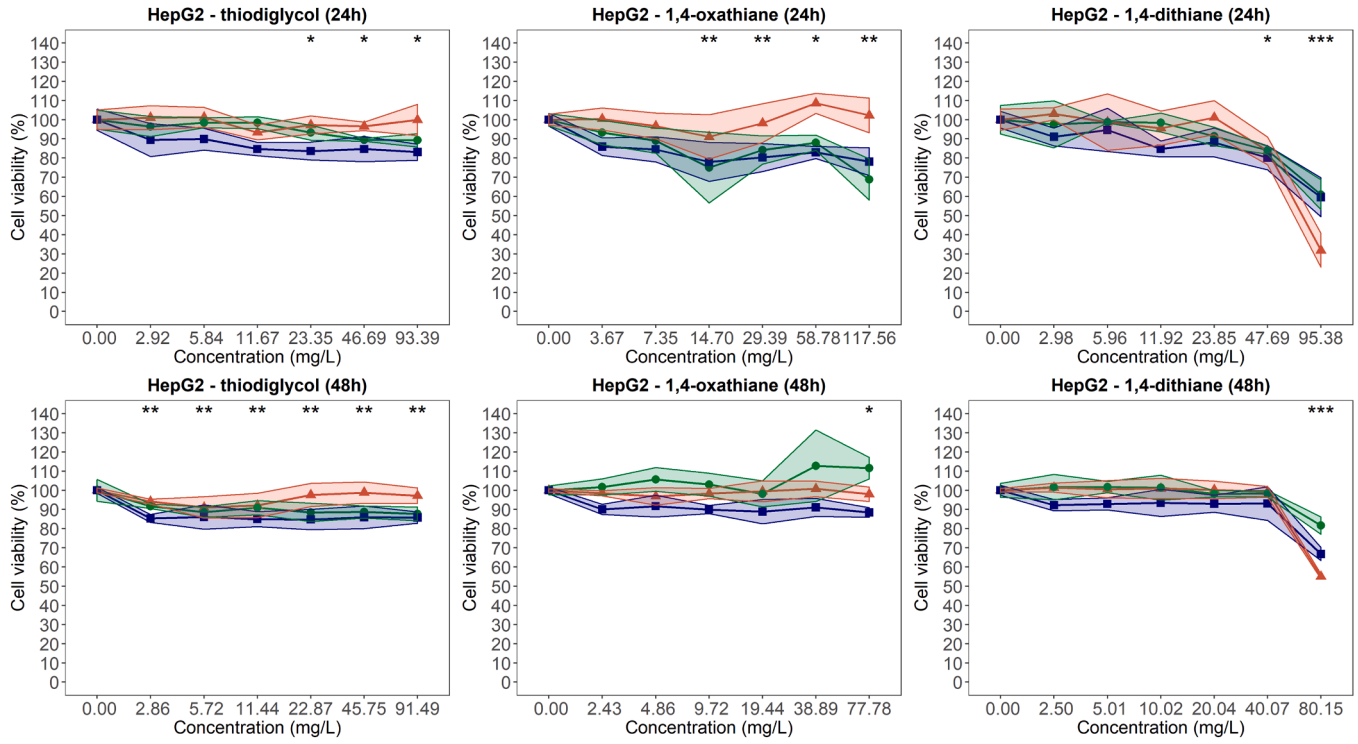


**Fig. 5.** Concentration-response curves of the tested CWA&RC for the three cell viability measures for HepG2, following 24 h and 48 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. * - $p < 0.05$, * * - $p < 0.01$, and * ** - $p < 0.001$.

1,3-DNB and picric acid (Goodfellow et al., 1983; van der Schalie, 1983). Specifically, van der Schalie et al. (1983) report a 96 h $LC_{50}$ for *Oncorhynchus mykiss* following exposure to 1,3-DNB of 1.7 mg/L, approximately ten times lower than that reported in this study, while a 96 h $LC_{50}$ for picric acid, for the same species, is reported at 110 mg/L, representing a value less than double the one reported here (Goodfellow et al., 1983). For TNT and tetryl, however, no directly comparable experimental data were found. The most comparable studies on these chemicals assessed the chronic impact of TNT in the survival of *Oncorhynchus mykiss* and resulted in the reporting of a lowest observed effect concentration (LOEC) of 0.49 mg/L following 60 days of exposure (Bailey et al., 1985), while a LOEC for *Sciaenops ocellatus* larval survival was set for tetryl at 1.1 mg/L after 2 days of exposure (Nipper et al., 2001). Notably, the estimated $EC_{10}$ and $EC_{50}$ values for tetryl and $EC_{10}$ value for 1,3-DNB are in the range of low hundreds of μg/L, well within the concentrations detected in environmental samples (Barton and Porter, 2004; Porter et al., 2011; Rodacy et al., 2001; USACE, 2013). When analyzing the assay sensitivity, it is clear that metabolic activity was the most sensitive to TNT, 1,3-DNB, and picric acid (see Fig. 2). A previous study showed that metabolic activity is overall the most sensitive of the endpoints in RTgill-W1, yet suggesting that the difference in assay sensitivity is associated with the chemicals' mode of action (Tanneberger et al., 2013). As no information is available on the mode of action of TNT, 1,3-DNB, and picric acid, no further conclusions can be drawn. Nonetheless, further research, focusing on lower biological levels, e.g. mitotoxicity or omics technologies, may provide an insight on the mechanisms initiating the cascade of events leading to the observed mortality (Müller et al., 2019). Given the current limitations on the understanding of the effects of CWA&RC and E&RC in fish and their importance in the marine food web, this would fill a major knowledge gap.

A similar rationale is applicable for the two human cell lines, Caco2 and HepG2, respectively representing acute toxicity to human colon and human liver. Interestingly, 1,4-oxathiane appears to more severely affect the human colon while 1,4-dithiane is the most toxic CWA&RC to the human liver, for which $EC_{50}$ values could be estimated after both 24 h and 48 h of exposure (see Table 2). When analyzing the toxic potential of the tested E&RC to both human cell lines, TNT and 1,3-DNB present much higher toxicity to Caco2 than to HepG2, while for tetryl and picric acid this is comparable between cell lines (see Table 2). One could argue that the lower sensitivity of HepG2 compared to Caco2 (here observed for thiodiglycol, 1,4-oxathiane, TNT, and 1,3-DNB) may be associated with hepatic clearance, however, recent studies have shown that HepG2 cells lack drug-metabolizing capacity (Stanley and Wolf, 2022). As such, differences in the observed toxicity only reflect different sensitivity between cell lines and, consequently, the lower sensitivity of the human liver when compared to the human colon cells. It is known that chronic exposure to TNT leads to severe health effects in humans, including anemia, spleen enlargement, disruption of the immune system, and abnormal liver functioning (Sanderson et al., 2017). While current human health hazard assessment guidelines, such as ECHA (2011), have not officially adopted *in vitro* tests to the assessment of acute toxicity, various cytotoxicity assays are under validation in an attempt to replace acute oral systemic tests (ECHA, 2011). Until then, the reported effects on human cell lines offer essential insight into the mechanisms of toxicity of these chemicals at cellular and subcellular endpoints, such as cellular structures, genes or proteins. When focusing on the underlying biological mechanisms leading to observed adverse outcomes in the liver using the AOPwiki (https://aopwiki.org/), the Key Event hepatotoxicity (KE 1393) is directly linked to the Adverse Outcome Pathway Cyp2E1 activation leading to liver cancer (AOP 220). The same AOP suggests that the activation of Cyp2E1 and oxidative stress happen prior to acute hepatotoxicity. That said, further tests targeting Cyp2E1 and oxidative stress may provide essential insight into the chemical concentrations at which these mechanisms are triggered. The same study by Sanderson et al. (2017) points out a significantly increased risk of colon and rectum cancer on the population of the island of Vieques between 1992 and 1997, an island used as a military test area

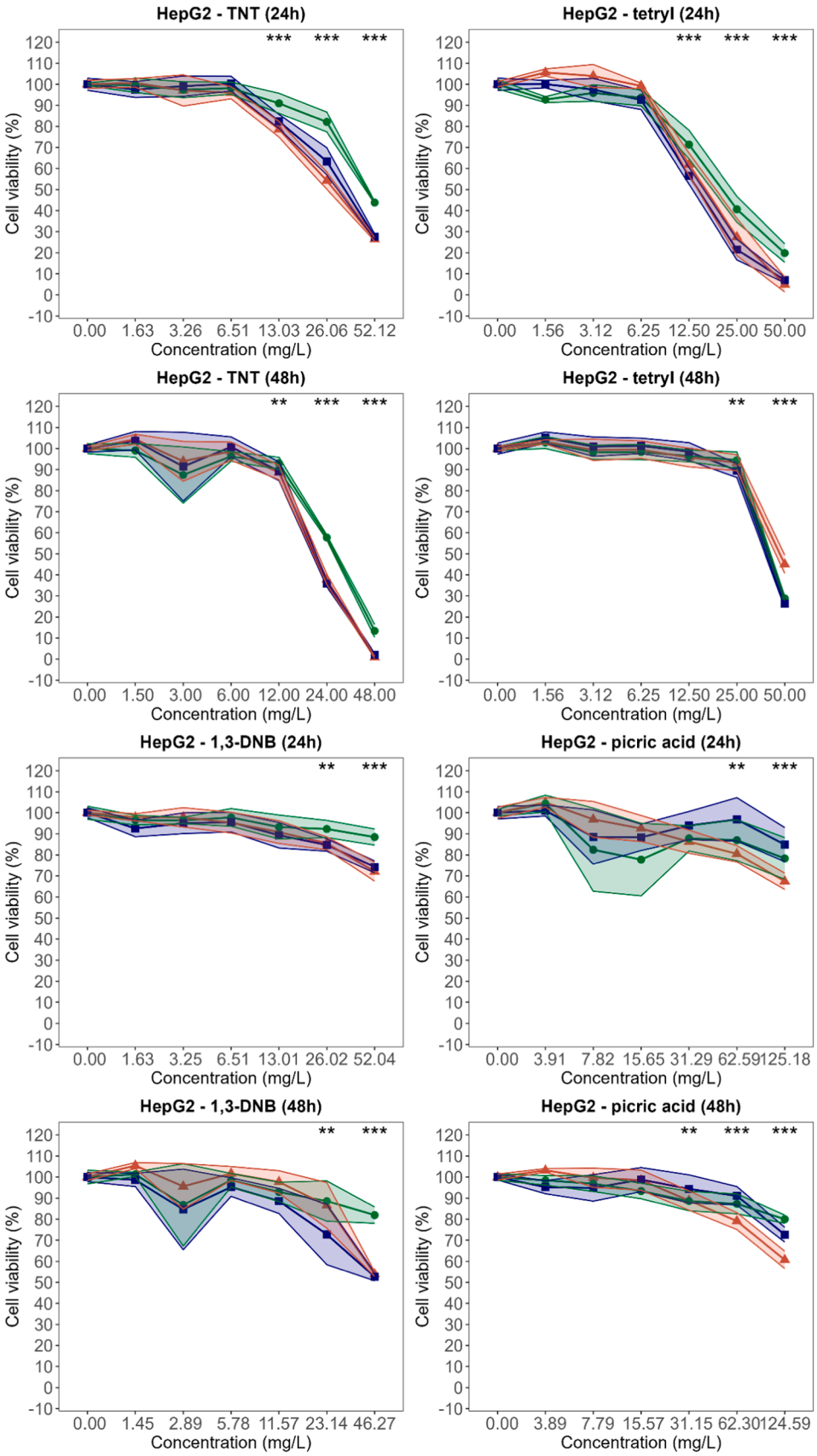


**Fig. 6.** Concentration-response curves of the tested E&RC for the three cell viability measures for HepG2, following 24 h and 48 h exposure, colored according to the different assays - blue represents alamarBlue, red NeutralRed and green CFDA-AM. Dots represent mean data with standard deviation shaded. *- $p < 0.05$, ** - $p < 0.01$, and ***- $p < 0.001$.

for more than six decades by the U.S. military (Sanderson et al., 2017). Regardless of the absence of an AOP directly linking damage to the human colon to colon cancer, AOP 205 provides a tentative pathway to the basal cytotoxicity observed for the tested cell lines, including Caco2. There, it is suggested that three distinct molecular events can initiate the cascade leading to cell death, i.e. cell decompartmentalization (MIE 1258), direct mitochondrial inhibition (MIE 1260), and narcosis (MIE 1259) hence providing information on targets for future assays.

To date, ecotoxicological studies have focused on assessing the impact of munition-related chemicals and their degradation products, particularly conventional explosives, at apical endpoints such as mortality (Koske et al., 2019; Nipper et al., 2001). Nonetheless, these fail to inform on how, and at which concentrations, the tested chemicals first affect organisms' fitness to the point of leading to the observed adverse outcomes. Moreover, despite the risk of chronic exposure via contaminated seafood, current knowledge on the impact of munition-related chemicals on human health is extremely scarce. That said, the previously discussed adverse outcome pathways provide insight into biological mechanisms potentially involved in the observed cytotoxicity (to fish, human colon and liver) that should be subject of further research. Supported by the constant advances in *in vitro* methodologies in the fields of ecotoxicology and human toxicology, further focus on understanding the effects at lower biological levels, rather than traditional apical endpoints, would help to unravel the underlying mechanisms of toxicity while offering essential information on potentially chronic effects to fish and human health.

## 5. Conclusion

The three tested degradation products of CWA&RC showed low acute cytotoxicity to fish (RTgill-W1), human colon (Caco2), and human liver (HepG2) cells. The tested degradation products of E&RC proved to be acutely more toxic than the degradation products of CWA&RC to the three cell lines. Of particular concern is the fact that the $EC_{10}$ and $EC_{50}$ values estimated for tetryl and $EC_{10}$ value for 1,3-DNB following exposure to RTgill-W1, here used as a proxy for fish acute toxicity, are comparable to concentrations detected in environmental samples collected in the vicinity of munition dumpsites over the North American east coast. Additionally, it should be kept in mind that while the estimated toxicity values provide important information on the impact of acute exposure to the seven tested chemicals, fish, and humans as seafood consumers, are chronically exposed to these chemicals. To that end, future lines of research were suggested.

The data here reported add to the current knowledge on the impact of relevant munition-related chemicals to fish and humans and outlines the need for further research at lower levels of biological complexity to shed light on the mechanisms involved in the observed toxicity.

## CRediT authorship contribution statement

**Geukens Kris:** Writing – review & editing, Validation, Resources. **François Philippe:** Writing – review & editing, Validation, Resources. **Asselman Jana:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Frédéric Laduron:** Formal analysis, Methodology. **Barbosa João:** Writing – original draft, Investigation, Formal analysis, Conceptualization. **Janssen Colin R:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Neyts Marijke:** Writing – review & editing, Validation, Resources. **Parmentier Koen:** Writing – review & editing, Validation, Resources.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.117867.

## Data availability

Data will be made available on request.

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