TEXTE
136/2025

Final report

Risk assessment of munition compounds in the German North and Baltic Sea (CONMAR-Impact)

Contribution to the CONMAR Project (CONcepts for conventional MArine Munition Remediation in the German North and Baltic Sea)

by:

Dr. Anita Künitzer, Christian Polleichtner, Dieter Schudoma

German Environment Agency (UBA), Dessau-Roßlau, Germany

Lillian T. H. Bünning

Institute of Toxicology and Pharmacology for Natural Scientists at the University Medical Center Schleswig-Holstein (UKSH), Kiel, Germany

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Publisher
Umweltbundesamt
Wörlitzer Platz 1
06844 Dessau-Roßlau
Germany
Tel: +49 340-2103-0
Fax: +49 340-2103-2285
buergerservice@uba.de
Internet: www.umweltbundesamt.de

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German Environment Agency
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Edited by:
Section II 2.3 Protection of the Seas and Polar Regions
Dr. Anita Künitzer

Layout by:
Section II 2.3 Protection of the Seas and Polar Regions
Anne Pampel

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Abstract: Risk assessment of munition compounds in the German North and Baltic Sea (CONMAR-Impact)

The sub-project 'CONMAR-Impact' comprised the following tasks within the framework of 'CONMAR':

- Work Package (WP)4 'Ecological and biological investigations in reference and munitions dumping areas', Task 4.5 'Laboratory exposure: impact of munition compounds (MC) on metabolism and health
- WP6 'Toxicological risk assessment of munitions in the sea', Task 6.1 'Eco-toxicological risk assessment

Task 4.5 'Laboratory exposure: impact of munition compounds (MC) on metabolism and health' is one of the two core tasks of the UBA in CONMAR-Impact: Tests were carried out to determine the ecotoxicity of TNT and its metabolites as well as other MC from the list of 13 relevant MC with 2 marine diatom species (*Phaeodactylum tricornutum* and *Skeletonema marinoi*). Dose-response relationships were determined in mono-species tests in accordance with DIN EN ISO 10253 (2016) and effect concentrations were derived on this basis.

The results show that all munition compounds and metabolites tested exhibited toxicity to the diatoms used. *S. marinoi* exhibited significantly higher sensitivity to TNT compared to *P. tricornutum*. Overall, TNT, followed by its metabolites 2-ADNT and 4-ADNT, exhibited the highest ecotoxicological potential of all munition compounds tested in this study. 1,3-DNB exhibited similar toxicity to the TNT metabolites 2-ADNT and 4-ADNT. The toxicity of 2,6-DNT was approximately one order of magnitude lower. According to literature data, RDX and HDX only become toxic to aquatic primary producers at concentrations in the high mg/L range and could not be tested in these concentration ranges due to their very low water solubility. The results were statistically compared in a species sensitivity distribution (SSD) with the SSDs of Lotufo et al. (2017). The comparison showed that the diatoms studied by the UBA are among the most sensitive species to MC tested to date.

The toxicological data were stored in the UBA's ETOX database. The ETOX database was migrated to the UBA's federal/state database ChemInfo in 2024 and the data are available there.

Task 6.1 "Ecotoxicological Risk Assessment" is UBA's second core task in CONMAR-Impact: Environmental quality standards (EQSs) were derived in CONMAR-Impact as ecotoxicologically derived limit values for TNT in water, sediment, and biota, as applied for the Water Framework Directive and the Marine Strategy Framework Directive.

Due to the extensive nature of the work, EQSs were derived only for TNT for water, sediment, biota, and humans. Because the toxicity of 2-ADNT and 4-ADNT is very similar to that of TNT, their environmental concentrations could be assessed additively with TNT under the EQS for TNT. Data searches were conducted in substance and literature databases, research reports for results of ecotoxicity and toxicity tests, and relevant data on environmental behaviour, such as water solubility, partition coefficients, bioconcentration factor, and degradation. The quality assessment of the effect data (CRED) and identification of key studies were carried out according to Moermond et al., 2016: test method, test substance, test organism, exposure conditions, statistical methods, biological effect (dose-response relationship).

As part of the data sheet preparation, quality standards (annual mean, AA-QSsw, eco and maximum value MAC-QSsw, eco) were derived (calculated) and justified for the individual protected assets (pelagic and benthic communities in freshwater, brackish water, or marine water, end links of the aquatic food chain (otters, common terns), human health via fish

consumption and drinking water). The most sensitive protected assets were identified, and a proposal for several cross-protected EQSs was developed. The peer review of the data sheet and the proposed quality standards was carried out by the REACH experts of the Federal Environment Agency and the Trace Substances Center at the Federal Environment Agency.

A proposal for the matrix (water, sediment, biota) for monitoring the EQS (most sensitive protected asset) was made after comparing the EQS with monitoring data. Since no EQS could be derived for sediment and concentrations in seawater are below the EQS, biota could be the preferred method of measurement.

Kurzbeschreibung: CONMAR-Impact: Risikobewertung von sprengstofftypischen Verbindungen (STV) in der deutschen Nord- und Ostsee

Das Teilprojekt „CONMAR-Impact“ umfasste im Rahmen von „CONMAR“ folgende Aufgaben:

- Arbeitspaket (AP)4 „Ökologische und biologische Untersuchungen in Referenz- und Munitions-versenkungsgebieten“, Aufgabe 4.5 „Laborexposition: Auswirkung von sprengstofftypischen-Verbindungen (STV) auf den Metabolismus und die Gesundheit“
- AP6 „Toxikologische Risikobewertung von Munition im Meer“, Aufgabe 6.1 „Öko-toxikologische Risikoabschätzung“

Die Aufgabe 4.5 „Laborexposition: Auswirkung von STV auf den Metabolismus und die Gesundheit“ ist eine der beiden Kernaufgaben des UBA in CONMAR-Impact: Es wurden Versuche zur Bestimmung der Ökotoxizität von TNT und seinen Metaboliten sowie weiteren STV aus der Liste der 13 relevanten STV mit 2 marinen Kieselalgenarten (*Phaeodactylum tricornutum* und *Skeletonema marinoi*) durchgeführt. Dabei wurden in Monospeziestests gemäß DIN EN ISO 10253 (2016) Dosis-Wirkungsbeziehungen bestimmt und auf deren Basis Effektkonzentrationen abgeleitet.

Die Ergebnisse zeigen, dass alle untersuchten STV und Metaboliten eine Toxizität gegenüber den verwendeten Kieselalgen aufwiesen. *S. marinoi* zeigte im Vergleich zu *P. tricornutum* eine deutlich höhere Empfindlichkeit gegenüber TNT. Insgesamt wies TNT, gefolgt von seinen Metaboliten, 2-ADNT und 4-ADNT, das höchste ökotoxikologische Potenzial aller in dieser Studie getesteten Munitionsverbindungen auf. 1,3-DNB zeigte eine ähnliche Toxizität wie die TNT-Metaboliten 2-ADNT und 4-ADNT. Die Toxizität von 2,6-DNT war etwa eine Größenordnung geringer. Nach Literaturdaten werden RDX und HDX erst bei Konzentrationen im hohen mg/L-Bereich für aquatische Primärproduzenten toxisch und konnten auf Grund ihrer sehr geringen Löslichkeit nicht in diesen Konzentrationsbereichen getestet werden. Die Ergebnisse wurden in einer Arten-Sensitivitätsverteilung (species sensitivity distribution, SSD) statistisch mit den SSDs von Lotufo et al. 2017 verglichen. Der Vergleich zeigte, dass die im UBA untersuchten Kieselalgen zu den empfindlichsten bisher getesteten Arten gegenüber STV gehören.

Die toxikologischen Daten wurden in der ETOX-Datenbank des UBA gespeichert. Die ETOX-Datenbank wurde 2024 in die Bund/Länder Datenbank ChemInfo des UBA migriert und die Daten sind dort abrufbar.

Die Aufgabe 6.1 „Öko-toxikologische Risikoabschätzung“ ist die zweite Kernaufgabe des UBA in CONMAR-Impact: Es wurden in CONMAR-Impact als ökotoxikologisch abgeleitete Grenzwerte für TNT in Wasser, Sediment und Biota Umweltqualitätsnormen abgeleitet, wie sie für die Wasserrahmenrichtlinie und Meeresstrategie-Rahmenrichtlinie Anwendung finden.

Da die Arbeiten sehr umfangreich waren, wurden UQN nur für TNT für Wasser, Sediment, Biota und den Menschen abgeleitet. Weil die Toxizität von 2-ADNT und 4-ADNT sehr ähnlich zur Toxizität von TNT ist, könnten deren Umweltkonzentrationen additiv mit TNT unter der UQN

für TNT bewertet werden. Datenrecherche wurde in Stoff- und Literaturdatenbanken, Forschungsberichten nach Ergebnissen von Ökotoxizitäts- und Toxizitätstests, relevanten Daten zum Umweltverhalten wie z.B. Wasserlöslichkeit, Verteilungskoeffizienten, Biokonzentrationsfaktor und Abbau durchgeführt. Die Qualitätsbewertung der Effektdaten (CRED) und Ermitteln der Schlüsselstudien erfolgte nach Moermond et al., 2016: Testmethode, Testsubstanz, Testorganismus, Expositionsbedingungen, statistische Methoden, biologische Wirkung (Dosis-Wirkungsbeziehung).

Im Rahmen der Datenblatterstellung wurden Qualitätsstandards (Jahresmittelwert, AA-QSsw, eco und Maximalwert MAC-QS sw, eco) für die einzelnen Schutzgüter (Pelagiale und benthische Lebensgemeinschaften in Süßwasser, Brackwasser oder Meerwasser, Endglieder der aquatischen Nahrungskette (Otter, Flussseeschwalbe), Gesundheit des Menschen via Fischkonsum und Trinkwasser) abgeleitet (berechnet) und begründet. Empfindlichste Schutzgüter wurden bestimmt und ein Vorschlag für mehrere schutzgutübergreifende Umweltqualitätsnormen erstellt. Der Peer Review des Datenblattes und der vorgeschlagenen Qualitätsstandards erfolgte durch die REACH Experten des Umweltbundesamtes sowie das Spurenstoffzentrum am Umweltbundesamt.

Ein Vorschlag für die Matrix (Wasser, Sediment, Biota) zur Überwachung der UQN (empfindlichstes Schutzgut) wurde nach Vergleich der UQN mit Monitoringdaten gemacht. Da keine UQN für Sediment abgeleitet werden konnte und die Konzentrationen im Meerwasser unterhalb der UQN liegen, sollte am ehesten in Biota gemessen werden.

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Abbreviation	Explanation
AA-EQS	Annual average standard
AA-QS$_{freshwater, eco}$	Annual average quality standard for freshwater community
AA-QS$_{marine water, sed.}$	Annual average quality standard for saltwater pelagic community
ACN	Acetonitrile
ADI	Acceptable daily intake
2-ADNT	2-Amino-2,6-dinitrotoluene
4-ADNT	4-Amino-2,6-dinitrotoluene
AF	Assessment factor
BCF	Bioconcentration factor
BLANO	Bund/LänderArbeitsgemeinschaft Nord- und Ostsee
CONMAR	CONcepts for conventional MArine Munition Remediation in the German North and Baltic Sea
CONMAR-Impact	Risk assessment of munition compounds in the German North and Baltic Seas
DAM	German Alliance for Marine Research
1,3-DNB	1,3-Dinitrobenzene
2,6-DNT	2,6-Dinitrotoluene
DOC	Dissolved Organic Carbon
EC$_x$	Effect Concentration at which x% effect is observed
EC$_{50}$	Concentration of an investigated substance that causes a certain toxicological effect in 50 % of the exposed organisms within a specified exposure periodE
ErC$_x$	Concentration of the test substance which results in an x % (e.g. 50%) reduction in growth rate relative to the negative control within a specified exposure period
EI	Electron Ionization
EqP	Equilibrium Partitioning
EQS	Environmental Quality Standard
GC-MS	Gas Chromatography-Mass Spectrometry
Growth rate	Growth rate refers to how quickly an organism or population increases in size, mass, or number over time. It's a key endpoint in ecotoxicology, used to assess the impact of a contaminant on living organisms.
HASEC	Hazardous Substances and Eutrophication Committee
HC$_5$	Hazardous concentration for 5% of species in a SSD
HELCOM	Helsinki Commission for the protection of the Baltic Sea
HMX	Abbreviation of “High Melting Explosive” (Octogen)

Abbreviation	Explanation
Kd	ratio of the concentration of a contaminant in the sediment to its concentration in water at equilibrium
K_{oc}	Organic carbon-water partition coefficient
K_{ow}	Octanol-water partition coefficient
LC_X	Leathal Concentration
LC_{50}	Concentration of an investigated substance that causes dead in 50 % of the exposed organisms within a specified exposure time
LOAEL	Lowest observed adverse effect level
LOEC	Lowest Observed Effect Concentration
MAC-EQS	Maximum allowable concentration standard
$MAC_{freshwater, eco}$	Maximum acceptable concentration for freshwater (short term)
$MAC_{saltwater, eco}$	Maximum acceptable concentration for saltwater (short term)
MC	Munition Compounds
MSFD	Marine Strategy Framework Directive
MTR	Maximum tolerable risk
n.d.	Not determined due to mathematical reasons or inappropriate data.
NOEC	No Observed Effect Concentration
OGewV	Oberflächengewässerverordnung
OSPAR	Oslo and Paris Commission for the protection of the North East Atlantic
$QS_{biota, hh, food}$	Quality standard for humans for the protection against adverse health effects from consuming fisheries products
$QS_{biota, secpoi}$	Quality standard for biota to protect against secondary poisoning of predators
R^2	Coefficient of Determination
R^2_{TNT}	Coefficient of Determination for TNT measurement
REMARCO	Remediation, Management, Monitoring and Cooperation addressing North Sea UXO
RFU	Relative Fluorescence Units
RDX	Abbreviation of "Research Department eXplosive" (Hexogen)
ROS	Reactive Oxygen Species
SAG	Culture Collection of Algae at the University of Göttingen, Germany
SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SSD	Species Sensitivity Distribution

Abbreviation	Explanation
SSL	Split Straight Liner
TDI	Tolerable Daily Intake
TGD-EQS	Technical Guidance Document for Deriving Environmental Quality Standards
TNT	2,4,6-trinitrotoluene
TSQ	Triple Quadrupole
UBA	Umweltbundesamt German Environment Agency
UKSH	Institute of Toxicology and Pharmacology for Natural Scientists at the University Medical Center Schleswig-Holstein (UKSH), Kiel, Germany
WFD	Water Framework Directive
Yield	Yield is used as a measure of biological productivity. It refers to population growth or biomass increase, measured as changing algae concentration under contaminant exposure. The reduction of yield might be a sign that a contaminant is causing harm to organisms and ecosystems.

Summary

The CONMAR partner project under the German Alliance for Marine Research (DAM), addresses key topics of the federal research program MARE:N. Like many other pollutants, munition compounds (MC) negatively impact ecosystem function and biodiversity. The robustness of ecosystems in the face of the continuous input of these compounds into the water column and sediments is not yet well understood. The partner project has helped policymakers with solution-oriented management concepts and has been validated through stakeholder involvement.

The sub-project 'CONMAR-Impact' comprised the following tasks within the framework of 'CONMAR':

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- WP6 'Toxicological risk assessment of munitions in the sea', Task 6.1 'Eco-toxicological risk assessment'

Task 4.5 'Laboratory exposure: impact of munition compounds (MC) on metabolism and health' is one of the two core tasks of the German Environment Agency in CONMAR-Impact: Tests were carried out to determine the ecotoxicity of TNT and its metabolites as well as other MC from the list of 13 relevant MC with 2 marine diatom species (*Phaeodactylum tricornutum* and *Skeletonema marinoi*). Dose-response relationships were determined in mono-species tests in accordance with DIN EN ISO 10253 (2016) and effect concentrations were derived on this basis. The work included the following steps:

- Selection of MC: Ecotoxicological studies were conducted with five MC (TNT, 1,3-DNB, 2-ADNT, 4-ADNT, 2,6-DNT) and two marine diatom species.
- Procurement of the test substances and all necessary chemicals for conducting the ecotoxicological tests.
- Cultivation of the marine diatom species *P. tricornutum* and *S. marinoi* in the laboratory. – During the course of the experimental work, difficulties in cultivating *P. tricornutum* arose repeatedly and with increasing frequency. For unknown reasons, the growth rates in the *P. tricornutum* algal cultures fluctuated considerably between different experiments, which repeatedly led to invalid test results. Even repeated procurement of the algal culture from the SAG Göttingen failed to permanently resolve this problem. Experiments with modified growth media and seawater preparations (both natural and artificial) were also unsuccessful. Therefore, towards the end of the second project year, it was decided to conduct the experimental trials exclusively with *S. marinoi*. This species was suitable for obtaining valid test results throughout the entire project period. The growth rates of *S. marinoi* were consistently sufficient, without significant fluctuations. Furthermore, a comparison of both species to TNT showed that *S. marinoi* is more sensitive than *P. tricornutum*. It can be assumed that this finding can very likely be transferred to the TNT metabolites 2-ADNT and 4-ADNT.
- Conducting reference tests to ensure the integrity of the test method – to ensure the integrity of the test systems and to verify that the selected test organisms react to the test substances with the expected sensitivity, reference tests with the reference substance 3,4-dichlorophenol (3,4-DCP) were conducted prior to the ecotoxicological experiments. The

results of these reference tests were compared with the results of an international interlaboratory comparison test conducted as part of the validation of DIN EN ISO 10253 (results in Baudo (2015)), as well as with the results of reference tests previously conducted in the German Environment Agency's ecotoxicology laboratory. The international laboratory proficiency test used *Skeletonema costatum*. However, based on molecular biological comparative analyses and a subsequent taxonomic revision, the diatom previously known as *S. costatum* was reclassified as *S. marinoi* on March 2, 2022.

- Sampling and analysis of samples to determine the actual concentrations used (analysis was carried out with the assistance of the project partner UKSH, Kiel) – Samples of the algal suspensions of all tested concentrations were taken in each experiment at the start of exposure and after 72 hours of exposure. The samples were prepared for analysis by eluting through a chromatography column along with a standard. The solvent volume was then reduced in a rotary evaporator, and the samples were transferred to sample vessels. For selected samples, the biomass was also separated from the water phase and mechanically disrupted before being applied to the column.

- Statistical evaluation and interpretation of the test results – Dose-response relationships were derived and effect concentrations determined using the ToxRat Professional XT software, version 3.3.0. A 3-parameter normal CDF (cumulative distribution function) was chosen as the model. LOEC and NOEC were also determined using the ToxRat Professional XT program, version 3.3.0. After a test for normal distribution and a test for homogeneity of variance, significant differences between the various test batches compared to the untreated control were determined using a t-tests. Based on the significant differences determined, values for LOEC and NOEC could be established.

- The toxicological data were stored in the ETOX database of the German Environment Agency. The ETOX database was migrated to the federal/state database ChemInfo in 2024. All obtained toxicity data on MC can be accessed there and in the results chapter of this report via QR codes from the ChemInfo database.

- The results on MC toxicity to diatoms show that all MC and metabolites tested exhibited toxicity to the diatoms used. *S. marinoi* showed significantly higher sensitivity to TNT compared to *P. tricornutum*. Overall, TNT, followed by its metabolites, 2-ADNT and 4-ADNT, exhibited the highest ecotoxicological potential of all munition compounds tested in this study. 1,3-DNB exhibited similar toxicity to the TNT metabolites 2-ADNT and 4-ADNT. The 2,6-DNT toxicity was approximately one order of magnitude lower. According to literature data, RDX and HDX only become toxic to aquatic primary producers at concentrations in the high mg/L range and could not be tested in these concentration ranges due to their very low water solubility. The results were statistically compared in a species sensitivity distribution (SSD) with SSDs from Lotufo et al. (2017). The comparison showed that the tested diatoms are among the most sensitive species to MC studied to date.

Task 6.1, 'Ecotoxicological risk assessment,' is the second core task of the German Environment Agency in CONMAR-Impact: To this end, CONMAR-Impact derived environmental quality standards (EQS) as ecotoxicologically derived limit values for TNT in water, sediment and biota, as applied in the Water Framework Directive (WFD) and Marine Strategy Framework Directive (MSFD).

In order to assess the risk to organisms from contaminants in water and to set limit values for the assessment, a guideline for the derivation of environmental quality standards under the Water Framework Directive (WFD) was established at European level, the so-called Technical

Guidance Document 27 (European Commission 2018). The German Environment Agency had extracted the relevant ecotoxicological data for munition compounds from its own databases and the ECHA database of REACH in a preliminary analysis. This showed that there is few ecotoxicological data available for munition compounds and/or that the few data available are very old and in some cases invalid or cannot be validated and therefore cannot be used for an assessment. Further ecotoxicological studies were therefore carried out in WP4, and their results, along with other results from this project, were included in the ecotoxicological risk assessment and derivation of EQS. The EQS derived in the project could be proposed internationally by Germany for the assessment of munition compounds within the framework of the Marine Strategy Framework Directive.

Four different data sets were required to calculate the ecotoxicological risk of munition compounds:

1. Results from laboratory experiments with various organisms (fish, mussels, crustaceans, algae) in which the toxicological endpoints and the EC50 and LC50 values were determined (AP4)
2. Literature data on EC50, EC10 and NOEC values for aquatic or benthic species and LD50 and NOAEL values for mammals and birds for the derivation of a quality standard for biota (secondary poisoning).
3. Concentrations of munition compounds in the marine environment (biota, water and sediments) from AP4.

The tasks of the German Environment Agency included the following work to derive EQS for munition compounds:

- Selection of substances to be processed, team formation and preparation: As the work is very extensive, EQS were only derived for TNT for water, sediment, biota and humans. Since the toxicity of 2-ADNT and 4-ADNT is very similar to that of TNT, their environmental concentrations might be assessed additively with TNT by applying the EQS for TNT.

- Data research: Research in substance and literature databases, research reports on the results of ecotoxicity and toxicity tests, relevant data on environmental behaviour such as water solubility, distribution coefficients, bioconcentration factor and degradation.

- Data evaluation: Quality assessment of effect data (CRED) and identification of key studies according to Moermond et al., 2016: 'Test method, test substance, test organism, exposure conditions, statistical methods, biological effect (dose-response relationship)'.

- Dossier/data sheet creation: Quality standards (annual mean value, $AA\text{-}QS_{sw,\,eco}$ and maximum value $MAC\text{-}QS_{sw,\,eco}$) for the individual protected resources (pelagic and benthic communities in fresh water, brackish water or sea water, end members of the aquatic food chain (otter, common tern), human health via fish consumption and drinking water) were derived (calculated) and justified.

- The most sensitive protected resources were identified and a proposal for several environmental quality standards covering multiple protected resources was drawn up.

- Monitoring data on TNT was requested and provided.

- The data sheet and the proposed quality standards were peer reviewed by REACH experts from the German Environment Agency and the Trace Substances Centre at the Federal Environment Agency. The MSRL D8/D9 expert network will be asked to comment in the follow-up project.

- A proposal for the matrix (water, sediment, biota) for monitoring the EQS (most sensitive protected resource) was made after comparing the EQS with monitoring data. Since no EQS could be derived for sediment and the concentrations in seawater are below the EQS, measurements should preferably be undertaken in biota.

The aim of the follow-up project is to develop a national MSRL-D8 indicator for munition compounds within the framework of the Marine Strategy Framework Directive, to promote this internationally via OSPAR and HELCOM stakeholder structures, and to have the derived EQS for TNT assessed by the MSRL Expert Network on Contaminants D8/ D9 and, if accepted, to use it for the assessment of measured munition compounds concentrations in monitoring for the assessment of TNT. Furthermore, the work presented can be used by stakeholders to decide on the need to establish a legally binding EQS for TNT.

Zusammenfassung

Das Verbundprojekt CONMAR im Rahmen der Deutschen Allianz für Meeresforschung (DAM) greift zentrale Themen des Bundesforschungsprogramms MARE:N auf. Wie viele andere Schadstoffe wirken sich auch sprengstofftypische Verbindungen (STV) negativ auf die Funktion von Ökosystemen und die Artenvielfalt aus. Die Robustheit von Ökosystemen angesichts des kontinuierlichen Eintrags dieser Verbindungen in die Wassersäule und die Sedimente ist noch nicht gut erforscht. Das Verbundprojekt hat politischen Entscheidungsträgern mit lösungsorientierten Managementkonzepten geholfen und wurde durch die Einbeziehung von Stakeholdern validiert.

Das vom Umweltbundesamt durchgeführte Teilprojekt „CONMAR-Impact“ umfasste im Rahmen von „CONMAR“ folgende Aufgaben:

- Arbeitspaket (AP)4 „Ökologische und biologische Untersuchungen in Referenz- und Munitions-versenkungsgebieten“, Aufgabe 4.5 „Laborexposition: Auswirkung von sprengstofftypischen Verbindungen (STV) auf den Metabolismus und die Gesundheit“
- AP6 „Toxikologische Risikobewertung von Munition im Meer“, Aufgabe 6.1 „Öko-toxikologische Risikoabschätzung“

Die Aufgabe 4.5 „Laborexposition: Auswirkung von STV auf den Metabolismus und die Gesundheit“ ist eine der beiden Kernaufgaben des UBA in CONMAR-Impact: Es wurden Versuche zur Bestimmung der Ökotoxizität von TNT und seinen Metaboliten sowie weiteren STV aus der Liste der 13 relevanten STV mit 2 marinen Kieselalgenarten (*Phaeodactylum tricornutum* und *Skeletonema marinoi*) durchgeführt. Dabei wurden in Monospeziestests gemäß DIN EN ISO 10253 (2016) Dosis-Wirkungsbeziehungen bestimmt und auf deren Basis Effektkonzentrationen abgeleitet. Die Arbeiten beinhalteten im Einzelnen folgende Schritte:

- Auswahl der STV: Es wurden ökotoxikologische Untersuchungen mit 5 STV (TNT, 1,3-DNB, 2-ADNT, 4-ADNT, 2,6-DNT) und 2 marinen Kieselalgenarten durchgeführt.
- Beschaffung der Testsubstanzen sowie aller notwendigen Chemikalien zur Durchführung der ökotoxikologischen Versuche.
- Kultivierung der marinen Kieselalgenarten *P. tricornutum* und *S. marinoi* im Labor. – Im Verlauf der experimentellen Arbeiten traten immer wieder und mit zunehmender Häufigkeit Schwierigkeiten bei der Kultivierung von *P. tricornutum* auf. Aus unbekannten Gründen schwankten die Wachstumsraten in den Algenkulturen von *P. tricornutum* zwischen verschiedenen Versuchen stark, was immer wieder zu ungültigen (nicht validen) Versuchsergebnissen führte. Auch der wiederholte Bezug der Algenkultur von der SAG Göttingen konnte dieses Problem nicht nachhaltig lösen. Auch Versuche mit modifizierten Wachstumsmedien und Meerwasseransätzen (sowohl natürlich als auch künstlich) blieben erfolglos. Daher wurde gegen Ende des zweiten Projektjahres beschlossen, die experimentellen Versuche nur mit *S. marinoi* durchzuführen. Diese Art war geeignet, um während des gesamten Projektzeitraums gültige/valide Versuchsergebnisse zu erzielen. Die Wachstumsraten von *S. marinoi* waren durchweg ausreichend, ohne große Schwankungen. Zudem zeigte der Vergleich beider Arten mit TNT, dass *S. marinoi* empfindlicher ist als *P. tricornutum*. Es kann davon ausgegangen werden, dass sich diese Erkenntnis sehr wahrscheinlich auf die TNT-Metaboliten 2-ADNT und 4-ADNT übertragen lässt.
- Durchführung von Referenzprüfungen zur Sicherstellung der Integrität der Testmethode – Um die Integrität der Testsysteme sicherzustellen und zu überprüfen, dass die ausgewählten

Testorganismen mit der erwarteten Empfindlichkeit auf die Testsubstanzen reagieren, wurden vor den ökotoxikologischen Experimenten Referenztests mit der Referenzsubstanz 3,4-Dichlorphenol (3,4-DCP) gemäß DIN EN ISO 10253 (2016) durchgeführt. Die Ergebnisse dieser Referenztests wurden mit den Ergebnissen eines internationalen Laborvergleichstests verglichen, der im Rahmen der Validierung der DIN EN ISO 10253 durchgeführt wurde (Ergebnisse in Baudo (2015)), sowie mit den Ergebnissen von Referenztests, die zuvor in der Ökotoxikologielabor des UBA durchgeführt wurden. Im Rahmen des internationalen Ringversuchs wurde die Kieselalge *Skeletonema costatum* verwendet. Aufgrund molekularbiologischer Vergleichsanalysen und einer anschließenden taxonomischen Revision wurde die zuvor als *S. costatum* bekannte Kieselalge am 2. März 2022 als *S. marinoi* neu klassifiziert.

- Probenahme und Analyse der Proben zur Bestimmung der eingesetzten Realkonzentrationen (Analytik erfolgte mit Hilfe des Projektpartners UKSH, Kiel) – Proben der Algensuspensionen aller getesteten Konzentrationen wurden bei jedem Versuch zu Expositionsbeginn und nach 72-stündiger Exposition genommen. Für die Analytik wurden die Proben vorbereitet, indem sie zusammen mit einem Standard über eine Chromatographiesäule eluiert wurden Das Lösemittelvolumen wurde anschließend im Rotationsverdampfer reduziert und die Proben in Probengefäße abgefüllt. Für ausgewählte Proben wurde zusätzlich die Biomasse von der Wasserphase getrennt und vor dem aufbringen auf die Säule mechanisch aufgeschlossen.

- Statistische Auswertung und Interpretation der Testergebnisse – Mit der Software ToxRat Professional XT, Version 3.3.0 wurden Dosis-Wirkungsbeziehungen abgeleitet und Effektkonzentrationen bestimmt. Als Modell wurde eine 3-parametrige normal CDF (cumulative ditribution function) gewählt. LOEC und NOEC wurden ebenfalls mit dem Programm ToxRat Professional XT, Version 3.3.0 bestimmt. Nach einem Test auf Normalverteilung und einem Test auf Varianzhomogenität wurden signifikante Unterschiede der verschiedenen Prüfansätze im Vergleich zur unbehandelten Kontrolle mit Hilfe von t-Tests bestimmt. Auf Basis der ermittelten signifikanten Unterschiede konnten jeweils Werte für LOEC und NOEC festgelegt werden.

- Die toxikologischen Daten wurden in der ETOX-Datenbank des UBA gespeichert. Die ETOX-Datenbank wurde 2024 in die Bund/Länder Datenbank ChemInfo des UBA migriert. Alle gewonnenen Toxizitätsdaten zu STV sind dort abrufbar und im Ergebniskapitel dieses Berichts über QR-Codes aus der ChemInfo-Datenbank abrufbar.

- Die Ergebnisse zur STV-Toxizität an Kieselalgen zeigen, dass alle untersuchten STV und Metaboliten eine Toxizität gegenüber den verwendeten Kieselalgen aufwiesen. *S. marinoi* zeigte im Vergleich zu *P. tricornutum* eine deutlich höhere Empfindlichkeit gegenüber TNT. Insgesamt wies TNT, gefolgt von seinen Metaboliten, 2-ADNT und 4-ADNT, das höchste ökotoxikologische Potenzial aller in dieser Studie getesteten sprengstofftypischen Verbindungen auf. 1,3-DNB zeigte eine ähnliche Toxizität wie die TNT-Metaboliten 2-ADNT und 4-ADNT. Die Toxizität von 2,6-DNT war etwa eine Größenordnung geringer. Nach Literaturdaten werden RDX und HDX erst bei Konzentrationen im hohen mg/L-Bereich für aquatische Primärproduzenten toxisch und konnten auf Grund ihrer sehr geringen Wasserlöslichkeit nicht in diesen Konzentrationsbereichen getestet werden. Die Ergebnisse wurden in einer Arten Sensitivitätsverteilung (species sensitivity distribution, SSD) statistisch mit SSDs von Lotufo et al. (2017) verglichen. Der Vergleich zeigte, dass die getesteten Kieselalgen zu den empfindlichsten, bisher untersuchten Arten gegenüber STV gehören.

Die Aufgabe 6.1 „Ökotoxikologische Risikoabschätzung“ ist die zweite Kernaufgabe des Umweltbundesamtes in CONMAR-Impact: Dazu wurden in CONMAR-Impact als ökotoxikologisch abgeleitete Grenzwerte für TNT in Wasser, Sediment und Biota Umweltqualitätsnormen (UQN) abgeleitet, wie sie für die Wasserrahmenrichtlinie (WRRL) und Meeresstrategie-Rahmenrichtlinie (MSRL) Anwendung finden.

Um die Gefährdung von Organismen durch Schadstoffe im Wasser abzuschätzen und Grenzwerte für die Bewertung festzulegen, wurde auf europäischer Ebene ein Leitfaden für die Ableitung von Umweltqualitätsnormen unter der Wasserrahmenrichtlinie (WRRL) festgelegt, das s.g. Technical Guidance Document 27 (European Commission 2018). Das Umweltbundesamt hatte aus seinen eigenen Datenbanken und der ECHA-Datenbank von REACH in einer Voranalyse die entsprechenden ökotoxikologischen Daten für STV extrahiert. Dabei zeigte sich, dass für STV kaum ökotoxikologische Daten vorliegen und/oder die wenigen vorhandenen Daten sehr alt sind sowie teilweise nicht valide oder nicht validierbar sind und daher nicht für eine Bewertung verwendet werden können. Es wurden daher im AP4 weitere ökotoxikologische Untersuchungen durchgeführt und deren sowie weitere Ergebnisse dieses Projekts in die ökotoxikologische Risikobewertung und Ableitung von UQN einbezogen. Die im Projekt abgeleiteten UQN können im Rahmen der Meeresstrategie-Rahmenrichtlinie international von Deutschland für die Bewertung von STV vorgeschlagen werden.

Für die Berechnung des ökotoxikologischen Risikos von STV wurden vier verschiedene Datensätze benötigt:

1. Ergebnisse aus Laborexperimenten mit verschiedenen Organismen (Fische, Muscheln, Krebstiere, Algen), in denen die toxikologischen Endpunkte sowie die EC_{50} und LC_{50}-Werte bestimmt wurden (AP4)
2. Literaturdaten zu EC_{50}-, EC_{10}- und NOEC-Werten für aquatisch oder benthisch lebenden Arten sowie LD_{50}- und NOAEL-Werte für Säuger und Vögel für die Ableitung eines Qualitätsstandards für Biota (secondary poisoning).
3. Konzentrationen von STV in der Meeresumwelt (Biota, Wasser und Sedimenten) aus AP4.

Die Aufgaben des Umweltbundesamtes beinhalteten folgende Arbeiten zur Ableitung von UQN für STV:

- Auswahl der zu bearbeitenden Stoffe, Teambildung und Vorbereitung: Da die Arbeiten sehr umfangreich sind, wurden UQN nur für TNT für Wasser, Sediment, Biota und den Menschen abgeleitet. Da die Toxizität von 2-ADNT und 4-ADNT sehr ähnlich zur Toxizität von TNT ist, könnten deren Umweltkonzentrationen additiv mit TNT unter der UQN für TNT bewertet werden.

- Datenrecherche: Recherche in Stoff- und Literaturdatenbanken, Forschungsberichten nach Ergebnissen von Ökotoxizitäts- und Toxizitätstests, relevanten Daten zum Umweltverhalten wie z.B. Wasserlöslichkeit, Verteilungskoeffizienten, Biokonzentrationsfaktor und Abbau.

- Datenbewertung: Qualitätsbewertung der Effektdaten (CRED) und Ermitteln der Schlüsselstudien nach Moermond et al., 2016: „Testmethode, Testsubstanz, Testorganismus, Expositionsbedingungen, Statistische Methoden, Biologische Wirkung (Dosis-Wirkungsbeziehung)“.

- Dossier-/Datenblatterstellung: Qualitätsstandards (Jahresmittelwert, $AA\text{-}QS_{sw,\,eco}$ und Maximalwert $MAC\text{-}QS_{sw,\,eco}$) für die einzelnen Schutzgüter (pelagiale und benthische Lebensgemeinschaften in Süßwasser, Brackwasser oder Meerwasser, Endglieder der aquatischen Nahrungskette (Otter, Flussseeschwalbe), Gesundheit des Menschen via Fischkonsum und Trinkwasser) wurden abgeleitet (berechnet) und begründet.

- Empfindlichste Schutzgüter wurden bestimmt und ein Vorschlag für mehrere schutzgutübergreifende Umweltqualitätsnormen erstellt.
- Monitoringdaten zu TNT wurden abgefragt und bereitgestellt.
- Der Peer Review des Datenblattes und der vorgeschlagenen Qualitätsstandards erfolgte durch die REACH Experten des Umweltbundesamtes sowie das Spurenstoffzentrum am Umweltbundesamt. Das MSRL D8/D9 Expertennetzwerk wird im Folgeprojekt um Stellungnahme gebeten.
- Ein Vorschlag für die Matrix (Wasser, Sediment, Biota) zur Überwachung der UQN (empfindlichstes Schutzgut) wurde nach Vergleich der UQN mit Monitoringdaten gemacht. Da keine UQN für Sediment abgeleitet werden konnte und die Konzentrationen im Meerwasser unterhalb der UQN liegen, sollte am ehesten in Biota gemessen werden.

Ziel für das Folgeprojekt ist es, einen nationalen MSRL-D8 Indikator zu STV im Rahmen der Meeresstrategie Rahmenrichtlinie zu entwickeln bzw. dies über OSPAR und HELCOM Stakeholder Strukturen international voranzutreiben sowie die abgeleitete UQN für TNT durch das MSRL Expert Network on Contaminants D8/D9 begutachten zu lassen und bei Akzeptanz für die Bewertung der gemessenen STV-Konzentrationen im Monitoring zur Bewertung von TNT zu nutzen. Weiterhin können die vorgelegten Arbeiten von Stakeholdern zur Entscheidung über Notwendigkeit der Festlegung einer rechtsverbindlichen UQN für TNT genutzt werden.

1 Introduction

Coastal waters around the world are contaminated with munitions from the two world wars (World War I and World War II), with around 1.6 million tonnes of munitions alone in the German parts of the North and Baltic Seas. The distribution and condition of the munitions in German waters are not sufficiently known. In addition to the explosion and safety risk, these munitions contain cytotoxic, genotoxic and carcinogenic chemicals in combination with conventional explosives, chemical warfare agents and munition components. There is increasing interest in the investigation and disposal of underwater munitions due to environmental and health risks and the hazards associated with dredging and the increasing development of offshore infrastructure associated with aquaculture, wind farms, cables and oil or gas pipelines, as well as increasing shipping traffic in general.

The research mission “Protection and Sustainable Use of Marine Areas” of the German Alliance for Marine Research (DAM) finances the CONMAR (CONcepts for conventional MArine Munition Remediation in the German North and Baltic Sea) project. The CONMAR project aims to integrate existing and new datasets on historical marine munitions, to pool the expertise and knowledge of German marine science organisations, government agencies and the private sector to advance our scientific understanding of the role, fate and impact of marine munitions in the environment, and to offer policy solutions for monitoring and remediation measures in consultation with stakeholders. The German Environment Agency (UBA) is as CONMAR partner institution undertaking research on ecotoxicology of toxic substances contained in munition and is undertaking the risk assessment of these substances (CONMAR-Impact).

1.1 Ecotoxicity of munition compounds

The CONMAR-Impact (Risk assessment of munition compounds in the German North and Baltic Sea) project, which is presented in this report, aimed in CONMAR work package 4 at providing toxicity data (short-term and chronic) for various typical munition compounds based on growth tests with two marine diatom species. The obtained toxicity data should be stored in the UBA database ETOX and the German ChemInfo Database.

The toxicity of 2,4,6-trinitrotoluene (TNT) and its metabolites has been extensively studied due to its widespread use as an explosive and its environmental persistence. TNT is a potent toxicant, exhibiting harmful effects across various biological organisms, including aquatic species, terrestrial animals, and humans. As a nitroaromatic compound, TNT undergoes biotransformation in organisms, leading to the formation of several metabolites that can vary in their toxicity compared to the parent compound. These metabolites include hydroxylated derivatives, amino and nitroso forms, which can exhibit distinct toxicological profiles.

In aquatic environments, TNT has been found to exert significant acute and chronic toxic effects on organisms from all trophic levels (Beck et al. 2019, Maser & Strehse 2020, Strehse et al. 2017, Appel et al. 2018). The mechanisms of toxicity are complex and multifactorial, involving both direct interactions with cellular components and the induction of oxidative stress. TNT can interact with cellular membranes, proteins, and DNA, leading to disruptions in cellular processes such as enzyme function, protein synthesis, and DNA replication. This interaction often results in cellular damage, apoptosis, and alterations in growth and reproduction. Furthermore, TNT’s ability to induce the production of reactive oxygen species (ROS) is a key factor contributing to its toxicity. ROS generation can lead to lipid peroxidation, protein denaturation, and DNA damage, all of which play critical roles in the adverse effects of TNT on living organisms (Adomako-Bonsu et al., 2024; Hung-Yu et al. 2017).

The metabolites of TNT, particularly 2-ADNT and 4-ADNT, can also exhibit high toxicity (Lotufo, 2017). These metabolites are typically formed by reductive processes involving microbial or enzymatic action. The transformation of TNT into these amino derivatives enhances its ability to penetrate biological membranes and interact with cellular structures. The nitro group in these metabolites is often more reactive than in TNT, contributing to their increased toxicological potency. The toxicity of these metabolites is particularly evident in the disruption of cellular energy production and interference with the function of vital enzymes, including those involved in the mitochondrial electron transport chain.

TNT's toxicity is not limited to aquatic organisms but extends to terrestrial species as well (Haas & Thieme, 1996; Gogal Jr., Johnson et al. 2002). In mammals, TNT has been shown to affect multiple organ systems, including the liver, kidneys, and nervous system. Its toxic effects in mammals are often associated with its ability to induce haematological disorders, immune suppression, and liver damage. The liver is a major site of TNT metabolism, and the toxic effects observed in this organ are linked to the accumulation of TNT and its metabolites, which can lead to hepatocellular injury. The nervous system is another target, with studies indicating that TNT exposure can lead to neurotoxic effects, including behavioural changes, cognitive deficits, and damage to neural tissue.

1.2 Species Sensitivity Distributions

Species Sensitivity Distributions (SSDs) are used as statistical method to assess the sensitivity of different species to a particular environmental stressor or pollutant. They are used in this study to relate the investigated toxicity of diatoms to the toxicity of other tested species.

SSDs have proven to be a very useful tool for certain applications:

1. **Environmental Risk Assessment:** SSDs are frequently used in ecological risk assessments to understand the impact of pollution on various species. Specifically, SSDs are used to estimate the **threshold probability** of harmful effects occurring at a specific pollutant concentration. For instance, the concentration at which 5% of the most sensitive species would be negatively impacted is determined (this is referred to as the **HC_5**, or 5th percentile concentration).
2. **Setting Environmental Standards and Thresholds:** In environmental policy and regulation, SSDs help establish **safety factors** that are used to set pollutant concentration limits in water, air, or soil. For example, if the HC_5 for a given chemical is determined at a specific concentration, this value might be used as a basis for regulatory standards.
3. **Ecological Protection Strategies:** SSDs enable the identification of the **protection needs** of the most sensitive species within an ecosystem and the development of targeted mitigation strategies. By assessing species sensitivities, measures can be taken to minimize the impact of pollutants and preserve biodiversity.
4. **Comparing Species Sensitivity:** SSDs provide a means to compare the sensitivities of different species to a given pollutant. This is particularly useful when considering species with different habitats, trophic levels, and ecological functions.
5. **Early Detection of Environmental Hazards:** By analysing SSDs, potential **environmental threats** from pollution can be identified at an early stage. This method helps to better understand the potential risk to the entire ecosystem based on toxicity data from individual species.

1.3 Environmental Quality Standards

Environmental quality standards (EQS) for the munition compounds should be derived and internationally tested in accordance with EU Technical Guidance Document No. 27 (European Comission 2018) in CONMAR work package 6. The EQS should be based on the obtained toxicity data and other toxicity data from partner organisations, the literature, as well as concentrations of munition compounds measured in CONMAR in various environmental media.

The EQS are planned to be used to assess the measured concentrations against these ecotoxicological limit values and trigger measures such as the recovery of ammunition where and when they are exceeded.

The EQS will be made available to the stakeholders of the Expert Group on Munitions in the Sea of the Federal/State Working Group on the North Sea and Baltic Sea (BLANO) for the creation of a national indicator on munition compounds. The national indicator could be expanded into an indicator for OSPAR and HELCOM.

The derivation of EQS is usually done under the Water Framework Directive (WFD) and follows a structured process that includes several scientific and regulatory steps:

1. Scientific research and risk assessment

Initially, a comprehensive scientific risk assessment is conducted to establish EQS. This assessment includes:

- Toxicity of substances:
  Studies are carried out to determine which concentrations of a pollutant may have harmful effects on the environment, such as on aquatic organisms (fish, insects, algae) or ecosystems.
- Exposure:
  The likelihood of a pollutant occurring at concentrations that could be harmful to the ecosystem is evaluated.
- Long-term effects:
  In many cases, long-term (chronic) impacts are considered, especially where a substance may cause damage at low concentrations over extended periods.

2. Setting of protection goals

The WFD sets overarching objectives for “Good ecological status” and “Good chemical Status” of water bodies. The EQS are derived from these objectives and are applied to a range of substances. These standards are intended to protect the health of aquatic ecosystems and maintain water quality for human use.

3. Derivation of thresholds

The derivation of environmental quality standards requires methodological guidelines. The principles are laid down in the TGD-EQS (European Commission, 2018):

- Effect-based thresholds:
  These are based on empirical data from scientific studies that describe the toxic effects of substances on aquatic organisms. Such thresholds, that should have no relevant effect are often expressed as EC_{10} values or No ObservedEffectConcentrations (NOEC) or a relevant effect LC_{50} or EC_{50} values.

- Tolerance Limits and Safety Margins:
  Safety factors or assessment factors are often incorporated to account for uncertainties in determining the risk threshold.

4. Consideration of environmental factors

The EQS can also consider environmental factors that may influence the toxicity of substances, such as pH, or chemical composition (water hardness or DOC). These variables can amplify or mitigate the effects of pollutants, so environmental quality standards may be modified by these parameters. This is particularly true for some metals such as cadmium, copper, and nickel.

5. Setting thresholds for specific substances

The European Commission establishes binding EQS for a range of pollutants, listed in an amending Directive to the WFD, the so called EQS-Directive (European Commission, 2008). These lists include both priority substances (particularly hazardous) and other pollutants that are also regulated. Each substance has specific concentration limits that cannot be exceeded in water bodies to ensure their "good ecological and chemical status".

6. Legal implementation and adjustment

Once established, the defined thresholds are transposed into national regulations by EU member states. They are regularly reviewed and updated based on new scientific findings to ensure that the standards reflect the current state of scientific knowledge. In Germany, the Oberflächengewässerverordnung OGewV, Anlage 8 (Bund, 2016) defines the EQS values for a number of hazardous substances which need to be regularly monitored.

To sum this up, the derivation of EQS involves scientific studies on the toxicity of pollutants, risk assessments to define thresholds for ecosystem damage, consideration of environmental factors, and the legal implementation of these standards through EU and national regulations. These standards contribute to the protection and long-term improvement of water bodies.

2 Materials and methods for toxicity tests with marine diatoms

The marine diatoms *Phaeodactylum tricornutum* (Bohlin, 1897) and *Skeletonema marinoi* (Sarno & Zingone, 2005) were used in this study. Both species were obtained from the culture collection of algae at the University of Göttingen (SAG), Germany.

P. tricornutum was cultivated in sterile artificial seawater and *S. marinoi* in natural seawater at 20 ± 1°C in a thermostatic chamber under continuous illumination with 70 ± 5 µmol photons m^{-2} s^{-1} for *S. marinoi*) and 90 ± 5 µmol photons m^{-2} s^{-1} for *P. tricornutum*, respectively (LI-COR LI-250A, LI-COR Environmental – GmbH).

P. tricornutum was constantly shaken at 100 rpm (Schüttler), whereas *S. marinoi* was shaken once every workday by hand. For *S. marinoi* shaking by hand was chosen over constant shaking because constant shaking would have disturbed the chain formation of the algae too much, especially at higher densities.

The cultures were grown in 100 mL Erlenmeyer-flasks each. The flasks were autoclaved for 20 min at 121°C filled with 25 mL of culture medium. Both cultures were diluted weekly.

Seawater was provided by the Alfred-Wegener-Institute, Helmholtz-Zentrum für Polar- und Meeresforschung (AWI), Bremerhaven, and had a salinity of 35 ppt. The culture medium was prepared according to the medium recipe for SWES-Medium Version 10.2008 provided by SAG.

Artificial seawater with a salinity of approximately 33 ppt was prepared according to DIN EN ISO 10253 (2016).

Table 1: Culture conditions of the two diatoms *P. tricornutum* and *S. marinoi*

Parameter	*P. tricornutum*	*S. marinoi*
Origin	SAG, Göttingen, Germany	SAG, Göttingen, Germany
Strain	SAG Nr. 1090-1a	SAG Nr. 19.99
Culture Medium	Sterile artificial seawater (salinity: 33 ppt)	Filtered natural seawater (salinity: 35 ppt; obtained from AWI, Bremerhaven)
Temperature	20 ± 1°C	20 ± 1°C
Illumination	90 ± 5 µmol photons m^{-2} s^{-1}	70 ± 5 µmol photons m^{-2} s^{-1}
Shaking	Constant shaking at 100 rpm	Shaken manually once per workday

Table 2: MA-Medium -- Synthetic sea water

Salt	Concentration of salt in synthetic sea water g/L
NaCl	22
$MgCl_2\ 6H_2O$	9.7
Na_2SO_4 (anhydrous)	3.7
$CaCl_2$ (anhydrous)	1.0
KCl	0.65
$NaHCO_3$	0.20
H_3BO_3	0.023

Source: DIN EN ISO 10253 (2016)

Table 3: MA-Medium -- Nutrient stock solutions

Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1		
$FeCl_3\ 6H_2O$	48 mg/L	149 µg/L (Fe)
$MnCl_2\ 4H_2O$	144 mg/L	605 µg/L (Mn)
$ZnSO_4\ 7H_2O$	45 mg/L	150 µg/L (Zn)
$CuSO_4\ 5H_2O$	0.157 mg/L	0.6 µg/L (Cu)
$CoCl_2\ 6H_2O$	0.404 mg/L	1.5 µg/L (Co)
H_3BO_3	1140 mg/L	3.0 mg/L (B)
Na_2EDTA	1000 mg/L	15.0 mg/L
Stock solution 2		
Thiamin hydrochloride	50 mg/L	25 µg/L
Biotin	0.01 mg/L	0.005 µg/L
Vitamin B12 (cyanocobalamin)	0.10 mg/L	0.05 µg/L
Stock solution 3		
K_3PO_4	3.0 g/L	3.0 mg/l; 0.438 mg/L P
$NaNO_3$	50.0 g/L	50.0 mg/l; 8.24 mg/L N
$Na_2SiO_3\ 5H_2O$	14.9 g/L	14.9 mg/l; 1.97 mg/L Si

Source: DIN EN ISO 10253 (2016)

2.1 Derivation of a correlation between cell concentration and fluorescence intensity

During the evaluation of a growth inhibition test with diatoms, the algal concentration must be determined in each test setup. The algal concentration and the fluorescence of the suspension are proportional; as the algal concentration increases, so does the fluorescence. Since directly counting the algae in all test setups is time-consuming, the algal concentrations were calculated based on the measured fluorescence.

Fluorescence measurements of diatom suspensions were conducted at an excitation wavelength of 440 nm and an emission wavelength of 680 nm using the same settings as listed under chapter 2.1 “Toxicity Tests”.

The correlation between algal concentration and fluorescence follows an approximately linear trend. To establish this relationship, a 6-step dilution series was prepared for both cultures, and fluorescence was recorded for each dilution step. The corresponding algal concentrations were determined by counting the number of cells under the microscope using a Neubauer Chamber.

A linear regression analysis was performed to derive the mathematical relationship between fluorescence intensity and algal concentration (cell number).

To optimize the fit of the regression, particularly in the lower ranges of fluorescence intensity and to minimize the occurrence of (theoretically possible) negative algal concentrations, the linear regression was fitted through the origin.

2.2 Reference Tests

Reference tests are generally necessary to ensure that the test organisms exhibit sufficient sensitivity for use in toxicity testing and to confirm that the test conditions and organisms are performing as expected.

This was done using the standard reference substance 3,5-dichlorophenol (Sigma-Aldrich) according to DIN EN ISO 10253 (2016). Both species were exposed to 8 different concentrations ranging from 0.5-10 mg/L for *P. tricornutum* and 0.3-7 mg/L for *S. marinoi* under the conditions described under chapter 2.3 “Toxicity Tests”.

The results were compared to literature values derived in an interlaboratory test (DIN EN ISO 10253 (2016)).

2.3 Toxicity Tests

Algal growth inhibition tests were performed according to DIN EN ISO 10253.

Briefly, exponentially growing algae were exposed to 7 - 10 different concentrations of each tested substance in triplicates or quadruplicates. All tests were conducted in 100 mL Erlenmeyer-flasks at 20 ± 1°C with continuous shaking at 100 rpm (*P. tricornutum*) or 30 rpm (*S. marinoi*) and continuous white light of 90-100 µmol photons m^{-2} s^{-1} (*P. tricornutum*) or 60-70 µmol photons m^{-2} s^{-1} (*S. marinoi*).

The initial cell densities were approximately 1×10^4 cells mL^{-1} for *P. tricornutum* and 2-5x 10^3 cells mL^{-1} for *S. marinoi* to account for the high but different growth rates of both species. Microalgal growth was measured using fluorescence (CLARIOstar® Plus, BMG Labtech GmbH) after 0, 24, 48, and 72 h. Each replicate was measured in duplicate in 96-well plates (Greiner Bio One™ F-Bottom 96-Well Assay Microplates) using a microplate reader (CLARIOstar® Plus, BMG Labtech GmbH). The excitation/emission wavelengths were 430/670 nm (9 nm bandwidth), and nine reads were obtained per well from the top. The microplate was shaken for 20 s in double-orbital mode before reading. In experiments with *S. marinoi* the initial cell density was too low to be measured for each concentration and replicate individually. Instead, the inoculate, which was prepared at a 10-fold concentration compared to the test solutions was used and the initial cell density was calculated based on those results.

A correlation between cell number and fluorescence was established prior to the toxicity tests for both species.

For the analysis 10 mL of each test concentration were collected as samples at the beginning and at the end of the exposure period. The replicates were combined prior to the 72-hour sampling. All samples were stored at −20°C until analysis.

The substances were tested in several range finding tests and in one or more definitive tests. The number of concentrations and replicates ranged from 7 to 10 concentrations and 3 to 4 replicates. During the whole period six control replicates were used and ran simultaneously to each test substance.

2.4 Statistical analysis

Statistical analysis was conducted in compliance with DIN EN ISO 10253 and OECD 201 test guideline using the statistic software ToxRat Professional, version 3.3.0 (ToxRat Solutions GmbH & Co. KG, Naheweg 15, 52477 Alsdorf, Germany).

The statistical design follows ToxRat's internal protocols:

Yield:

- Pre-testing for Normality: Shapiro–Wilk test ($\alpha = 0.01$)
- Pre-testing for Homogeneity of Variances: Levene's test ($\alpha = 0.01$)
- Monotonicity Check: Contrast Analysis ($\alpha = 0.05$)
- Final NOEC Determination: Williams test (one-sided, smaller)
  - Variance Used: Residual from ANOVA
  - Significance Level: $\alpha = 0.05$
- EC_X Computation:
  - Method: Non-linear regression using a three-parameter Normal function
  - Optimization Algorithm: Levenberg–Marquardt (IRLS)
  - Confidence Limits: Monte Carlo simulation

Growth Rate:

- Pre-testing for Normality: Shapiro–Wilk test ($\alpha = 0.01$)
- Pre-testing for Homogeneity of Variances: Levene's test ($\alpha = 0.01$)

- Monotonicity Check: Contrast Analysis (α = 0.05)
- Final NOEC Determination: Williams test (one-sided, smaller)
  - Variance Used: Residual from ANOVA
  - Significance Level: α = 0.05
- EC_X Computation:
  - Method: Non-linear regression using a three-parameter Normal function
  - Optimization Algorithm: Levenberg–Marquardt (IRLS)
  - Confidence Limits: Monte Carlo simulation

2.5 Tested compunds and stock solutions

Table 4: Tested compounds

name	short name	purity	size	manufacturer
2,4,6-Trinitrotoluene	TNT	1000 ± 6 µg/mL	1000 µg/mL in acetonitrile, ampule of 1.2 mL	Cerilliant
2-Amino-4,6-Dinitrotoluene	2-ADNT	99%	10 mg	Dr. Ehrenstorfer
4-Amino-2,6-Dinitrotoluene	4-ADNT	99.9%	100 mg	Cerilliant
1,3-Dinitrobenzene	1,3-DNB	≥98%	100 mg	Sigma-Aldrich
2,6-Dinitrotoluene	2,6-DNT	99.4%	250 mg	Dr. Ehrenstorfer

For the preparation of a TNT stock solution, nine 1.2 mL vials were combined, and two 5 mL aliquots were pipetted into test tubes. An RVC 2-25 CDplus rotary vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) was subsequently utilized to evaporate the acetonitrile. The remaining residue was then dissolved in approximately 80 mL dichloromethane and transferred into two 500 mL volumetric flasks. This portion of the preparation was conducted at the Institute of Toxicology and Pharmacology for Natural Scientists at the University Medical Center Schleswig-Holstein, UKSH Kiel, Germany.

Upon return to Berlin, the volumetric flasks were placed under a fume hood for 24 hours to allow the dichloromethane to evaporate. The remaining solvent was evaporated by immersing the flasks in a water bath at a temperature of up to 40°C for 4 hours.

The TNT was then dissolved in 500 mL of artificial seawater (*P. tricornutum*) or filtered natural seawater (*S. marinoi*).

The solutions were stirred continuously for approximately 2 hours, then heated in a water bath to a maximum of 40°C, stirred again for 30 min, and subsequently sonicated in 5-minute increments with intermittent stirring until complete dissolution was visually confirmed.

All other test substances were purchased in their crystalline forms and dissolved similarly to the process described for TNT.

After preparation all stock solutions were stored at 2°C until use.

2.6 Chemical Analysis

2.6.1 Sample preparation

Sample preparation was performed at the Institute of Toxicology and Pharmacology for Natural Scientists at the University Medical Center Schleswig-Holstein, UKSH Kiel, Germany.

CHROMABOND Easy polystyrene-divinylbenzene-copolymer reversed-phase solid-phase extraction columns 80 µm, 3 mL/200 mg (Macherey Nagel, Düren, Germany) were conditioned with 1 mL of 18.2 MΩ·cm water, 2 mL dichloromethane and again 1 mL 18.2 MΩ·cm water before use.

Samples were transferred into reservoirs and introduced through the conditioned columns using a mild vacuum (-80 mbar). The water was discarded, and the columns were dried under vacuum (1 h) before being eluted with 2 times 2 mL of acetonitrile (ACN). The eluates were concentrated to less than 1 mL using an RVC 2-25 CDplus rotary vacuum concentrator (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), made up to 1 mL with ACN and were transferred to amber 1.5 mL autosampler vials. Samples were stored at 20°C.

2.6.2 Analytical Measurement with GC-MS/MS

General parameters

The analysis was carried out using a TSQ 8000 EVO triple quadrupole mass spectrometer coupled with a TRACE 1310 gas chromatograph (GC-MS/MS) and a TriPlus100LS autosampler equipped with a cooling tray (4°C) (Thermo Fisher Scientific Inc.). TG-5MS AMINE columns (15m x 0.25 mm x 0.25 µm, Thermo Fisher Scientific Inc.) were employed for analyte separation. Sample injections were carried out into a split- splitless injector with deactivated glass wool injection port liners (4 mm x 6.3 mm x 78.5 mm, Thermo Fisher Scientific Inc.). Helium (99.999 % purity, Air Liquide Deutschland GmbH) was used as carrier gas, and argon (99.999 % purity, Air Liquide Deutschland GmbH) served as collision gas. Data acquisition and analysis were performed in Chromeleon 7.2.10 software (Thermo Fisher Scientific Inc.). All samples were analysed in duplicate. An injection volume of 1 µL was used for each measurement. To check measurement accuracy and consistency, a 100 ng/mL check standard was measured after every 10 samples. Negative controls were measured at first, even before calibration standards to avoid potential carry-over.

2.6.3 Measurement of 2,6-DNT

For the analysis of the 2,6-DNT samples a standard series with concentrations ranging from 10 to 10000 ng/mL was used. The dilutions were prepared in acetonitrile (ACN) from a 2,6-DNT stock solution with a concentration of 1 mg/mL from AccuStandard (New Haven, Conneticut, USA). Dilutions were freshly prepared for the measurement and stored at 4°C in the dark until use and during analysis. The obtained calibration curve exhibited excellent linearity, with a coefficient of determination (R^2) of 99.965 %.

Measurements was conducted in split mode with a split flow of 300 mL/min and a split ratio of 200. The injector temperature was set to 230°C, while the gas flow rate was 1.5 mL/min. The oven temperature program started at 100°C with a hold time of 0.2 minutes, followed by a ramp of 30°C/min to 220°C, where the temperature was held for 0.3 minutes. The total analysis time was 4.8 minutes. Detection was performed using tandem quadrupole mass spectrometry (MS/MS) with electron ionization (EI) in selected reaction monitoring (SRM) mode. SRM-parameters are given in Tab. 5.

Table 5: GC-MS/MS - parameters for 2,6-DNT

Name	Retention time [min]	Transition [m/z]	Collision energy [eV]	Used for
2,6-DNT	2.60	165.1 > 63.1	24	Quantification
2,6-DNT	2.60	165.1 > 90	14	Confirmation
2,6-DNT	2.60	165.1 > 119	6	Confirmation
2,6-DNT	2.60	89.1 > 63.0	12	Confirmation

2.6.4 Measurement of 2- and 4-ADNT

The analysis of the 2- and 4-ADNT samples was performed using a standard series with concentrations ranging from 10 to 10000 ng/mL. The dilutions were prepared in acetonitrile (ACN) from a 1 mg/mL stock solution from AccuStandard. All solutions were freshly prepared for the measurement and stored at 4°C in the dark until use and during analysis. The calibration curves showed excellent linearity, with R^2 values of 99.64 % for 4-ADNT and 99.52 % for 2-ADNT.

The measurement was conducted in split mode with a split flow of 75 mL/min and a split ratio of 50. The injector temperature was set to 230°C, and the gas flow rate was 1.5 mL/min. The oven temperature program started at 100°C with a hold time of 0.2 minutes, followed by an increase at 30°C/min to 220°C (held for 0.3 minutes), and then further increased at 80°C/min to 280°C, where it was held for 1 minute. The total analysis time was 6.25 minutes. Detection was performed using tandem quadrupole mass spectrometry (MS/MS) with electron ionization (EI) in selected reaction monitoring (SRM) mode. SRM-parameters are given in Tab. 6.

Table 6: GC-MS/MS - parameters for 2- and 4-ADNT

Name	Retention time [min]	Transition [m/z]	Collision energy [eV]	Used for
4-ADNT	4.92	197.0 > 180.1	14	Quantification
4-ADNT	4.29	163.0 > 78.0	8	Confirmation
4-ADNT	4.92	80.0 > 163.1	6	Confirmation
2-ADNT	5.13	197.0 > 180.1	12	Quantification
2-ADNT	5.13	180.0 > 67.0	6	Confirmation
2-ADNT	5.13	180.0 > 133.0	6	Confirmation

2.6.5 Measurement of 1,3-DNB

The measurement of high-concentration water samples was performed using a standard series ranging from 1000 to 100000 ng/mL. For the analysis of the post exposure water, a standard series from 10 to 1000 ng/mL was used, while algae samples were analyzed with concentrations between 10 and 5000 ng/mL. In all cases, dilutions were prepared in acetonitrile (ACN) from a 1 mg/mL stock solution from AccuStandard, freshly prepared for measurement, and stored at 4°C in the dark until use and during analysis. The calibration curves showed high linearity, with R^2

values of 99.711 % for high concentration water, 99.751 % for post exposure water, and 99.879 % for algae samples.

Measurements were conducted in split mode with different split flows: 75 mL/min and a split ratio of 50 for high concentration water, 8 mL/min and a split ratio of 5 for post exposure water, and 15 mL/min with a split ratio of 10 for algae samples. The injector temperature was set to 230°C, and the gas flow rate was 1.5 mL/min. The oven temperature program started at 100°C with a hold time of 0.2 minutes, followed by an increase at 30°C/min to 220°C, where it was held for 0.3 minutes. The total run time was 4.8 minutes. Detection was performed using tandem quadrupole mass spectrometry (MS/MS) with electron ionization (EI) in selected reaction monitoring (SRM) mode. SRM-parameters are given in Tab. 7.

Table 7: GC-MS/MS - parameters for 1,3-DNB

Name	Retention time [min]	Transition [m/z]	Collision energy [eV]	Used for
1,3-DNB	2.53	168.0 > 75.0	20	Quantification
1,3-DNB	2.53	122.0 > 75.0	12	Confirmation
1,3-DNB	2.53	168.0 > 122.0	8	Confirmation

2.6.6 Measurement of TNT

The measurements of water and algae samples with concentrations of 0.75–3.5 mg/L were performed using standards from 1000 to 25000 ng/mL. For the negative control and samples up to 0.5 mg/L, 50 to 1000 ng/mL were used. In both cases, dilutions were prepared in acetonitrile (ACN) from a 1 mg/mL stock solution from AccuStandard, freshly prepared for the measurement, and stored at 4°C in the dark until use and during analysis. The calibration curves demonstrated high linearity, with R^2 values of 99.39 % for TNT, 99.57 % for 4-ADNT, and 99.10 % for 2-ADNT in water and algae samples, while values of 99.77 % (TNT), 99.87 % (4-ADNT), and 99.76 % (2-ADNT) were obtained for algae NK-0.5 samples.

The measurements were conducted in split mode with different split flows: 75 mL/min and a split ratio of 50 for the samples with 0.75-3.5 mg/L, and 45 mL/min with a split ratio of 30 for the negative control to 0.5 mg/L samples. The injector temperature was set to 230°C, and the gas flow rate was 1.5 mL/min. The oven temperature program started at 100°C with a hold time of 0.2 minutes, followed by an increase at 30°C/min to 220°C (held for 0.3 minutes), and then further increased at 80°C/min to 280°C, where it was held for 1 minute. The total analysis time was 6.25 minutes. Detection was performed using tandem quadrupole mass spectrometry (MS/MS) with electron ionization (EI) in selected reaction monitoring (SRM) mode. SRM-parameters are given in Tab. 8.

Table 8: GC-MS/MS - parameters for TNT

Name	Retention time [min]	Transition [m/z]	Collision energy [eV]	Used for
TNT	3.50	210.0 > 164.1	6	Quantification
TNT	3.50	180.0 > 76.1	10	Confirmation
TNT	3.50	164.0 > 90.1	12	Confirmation

Name	Retention time [min]	Transition [m/z]	Collision energy [eV]	Used for
4-ADNT	4.33	197.0 > 180.1	14	Quantification
4-ADNT	4.33	163.0 > 78.0	8	Confirmation
4-ADNT	4.33	180.0 > 163.1	6	Confirmation
2-ADNT	4.54	197.0 > 180.1	12	Quantification
2-ADNT	4.54	180.0 > 67.0	6	Confirmation
2-ADNT	4.54	180.0 > 133.0	6	Confirmation

3 Results of toxicity tests of munition compounds with marine diatoms

3.1 Derivation of a correlation between cell concentration and fluorescence intensity

As described in the chapter 2 “Materials and Methods for toxicity tests with marine diatoms”, to perform time efficient toxicity tests with algae it is warranted to use so called surrogate parameters such as absorbance (optical density) or fluorescence intensities to determine the algae concentration instead of counting algae.

Therefore, a correlation between cell concentration and fluorescence intensity was determined for *P. tricornutum* and *S. marinoi*.

3.1.1 Phaeodactylum tricornutum

Table 9: Correlation between cell concentration and fluorescence intensity for *P. tricornutum*

sample	cell concentration [1/mL]	fluorescence intensity [RFU][1]
1	1298125	107340
2	663542	57796
3	331250	27319
4	136667	10240
5	10625	905
6	5625	456

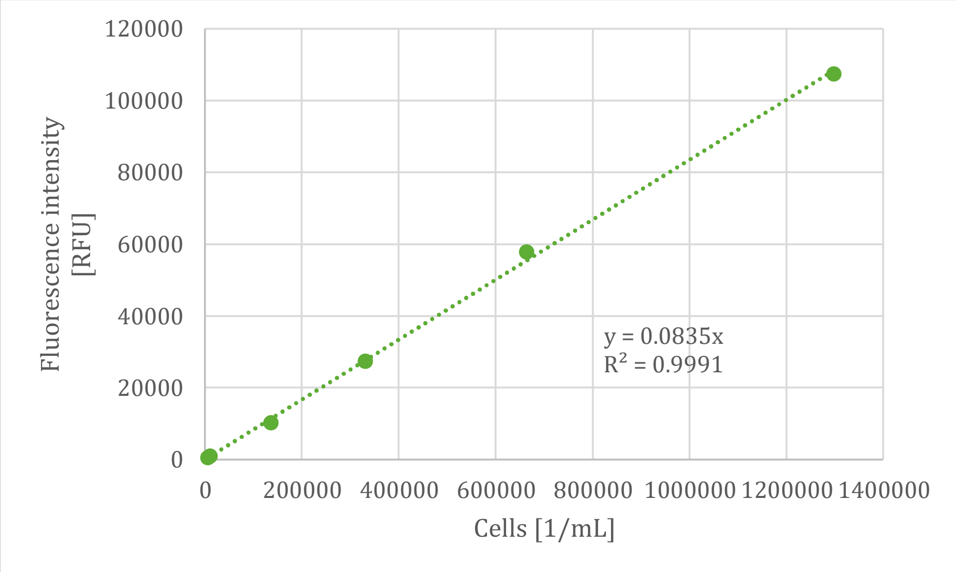
mathematical model	linear regression	y = ax + b
slope a = 0.0835 mL/cells	intercept b = 0	determination coefficient R^2 = 0.9991

$$y = 0.0835 \left[\frac{mL}{cells}\right] * x \left[\frac{cells}{mL}\right] + 0$$

All validity criteria according to DIN EN ISO 10253 (2016) were met with the correlations.

[1] Relative Fluorescence Units

Figure 1: Calibration line for correlation between cell concentration and fluorescence intensity *P. tricornutum*



Source: own illustration, German Environment Agency

3.1.2 Skeletonema marinoi

Table 10: Correlation between cell concentration and fluorescence intensity for *S. marinoi*

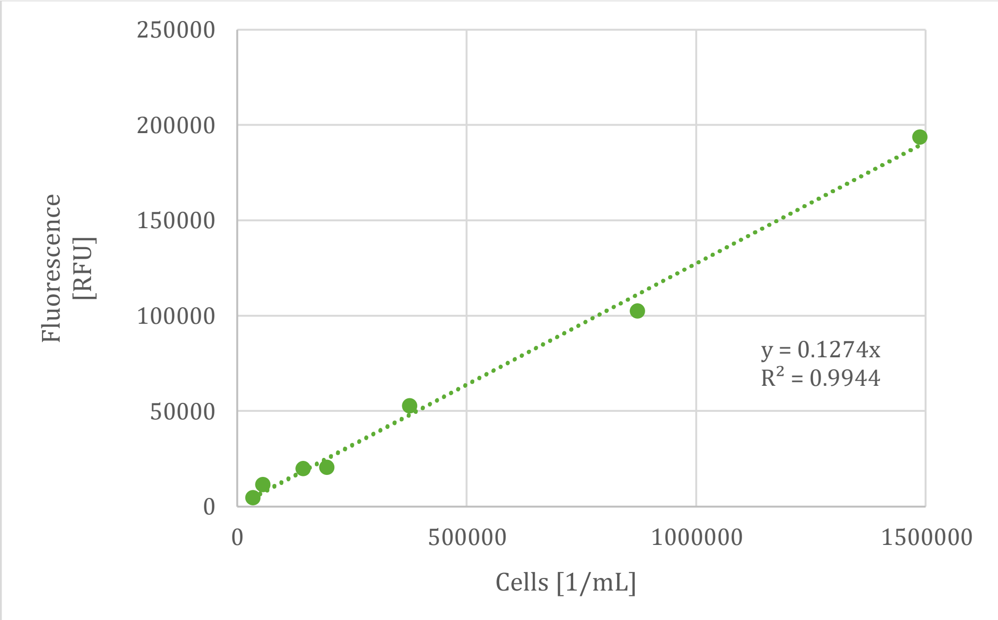
sample	cell concentration [1/mL]	fluorescence intensity [RFU]
1	1487500	144387
2	872500	76444
3	375833	39073
4	195833	14949
5	144167	15355
6	56667	8972

mathematical model:	linear regression	y = ax + b
slope a = 0.1274 mL/cells	intercept b = 0	determination coefficient R^2 = 0.9944

$$y = 0.1274 \left[\frac{mL}{cells}\right] * x \left[\frac{cells}{mL}\right] + 0$$

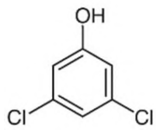
All validity criteria according to DIN EN ISO 10253 (2016) were met with the correlations.

Figure 2: Calibration line for correlation between cell concentration and fluorescence intensity *S. marinoi*



Source: own illustration, German Environment Agency

3.2 3,5 DCP (3,5-Dichlorophenol)



Reference testing

3.2.1 Phaeodactylum tricornutum

Schelzig, S. (2022a): Prüfung der Toxizität von 3,5-Dichlorphenol auf marine Kieselalgen der Art *Phaeodactylum tricornutum* (Referenztest no. C03Pt). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C03Pt.

https://recherche.chemikalieninfo.de/etox/zitat/124180?sv=

The results of the reference testing of the toxicity of 3,5 DCP on *Phaeodactylum tricornutum* are shown in the tables 11 and 12 and figures 3, 4, 5, 6 and 7.

Effect concentrations

Table 11: Effect concentrations for test item 3,5-DCP

EC_x [mg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	0.739	1.537
lower CL[2]	0.679	0.643
upper CL	0.805	3.671
EC_{50}	1.319	2.283
lower CL	1.196	0.820
upper CL	1.1457	6.266
LOEC	> 10.0	> 10.0
NOEC	≥ 10.0	≥ 10.0

Inhibition of yield and growth rate

Table 12: Inhibition of yield and growth rate over 72 hours by test item 3,5-DCP

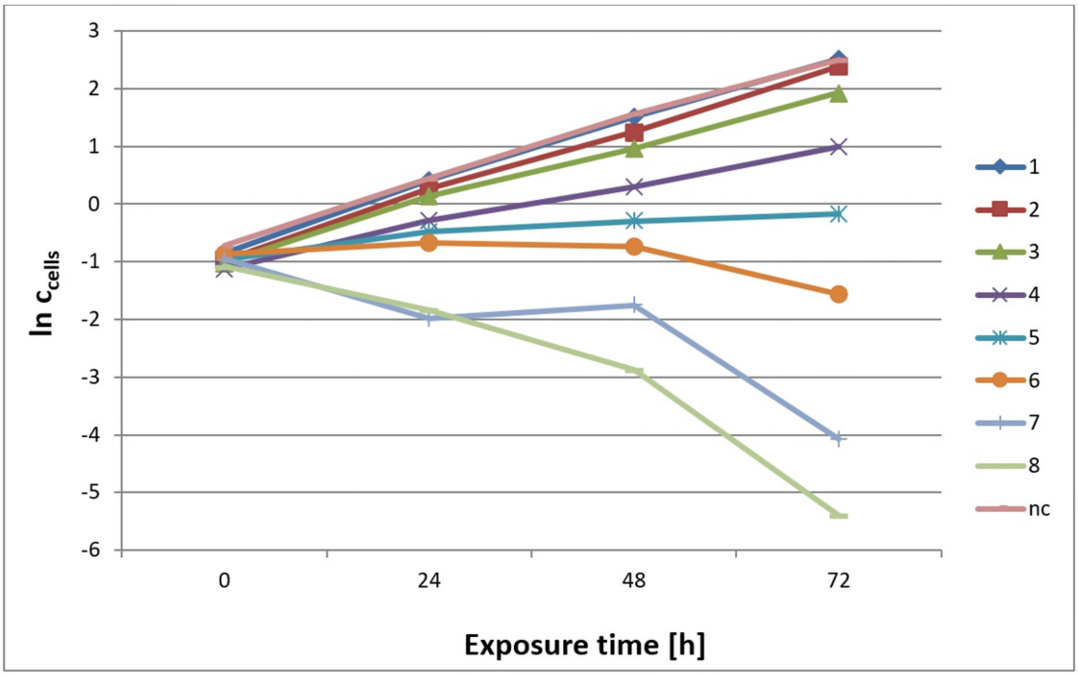
sample	test item concentration[3] [mg/L]	inhibition [%] yield	inhibition [%] growth rate
nc[4]	0	-	-
1	0.50	-3.1	-5.3
2	0.80	9.6	-5.2
3	1.20	44.1	8.0
4	2.00	79.5	33.6
5	3.00	96.0	75.4
6	4.50	101.8	122.8
7	7.00	103.2	199.8
8	10.0	102.5	176.0

[2] 95 % confidence level
[3] nominal
[4] negative control; no treatment with test item

Dose-response relationships

Cell concentration over exposure time

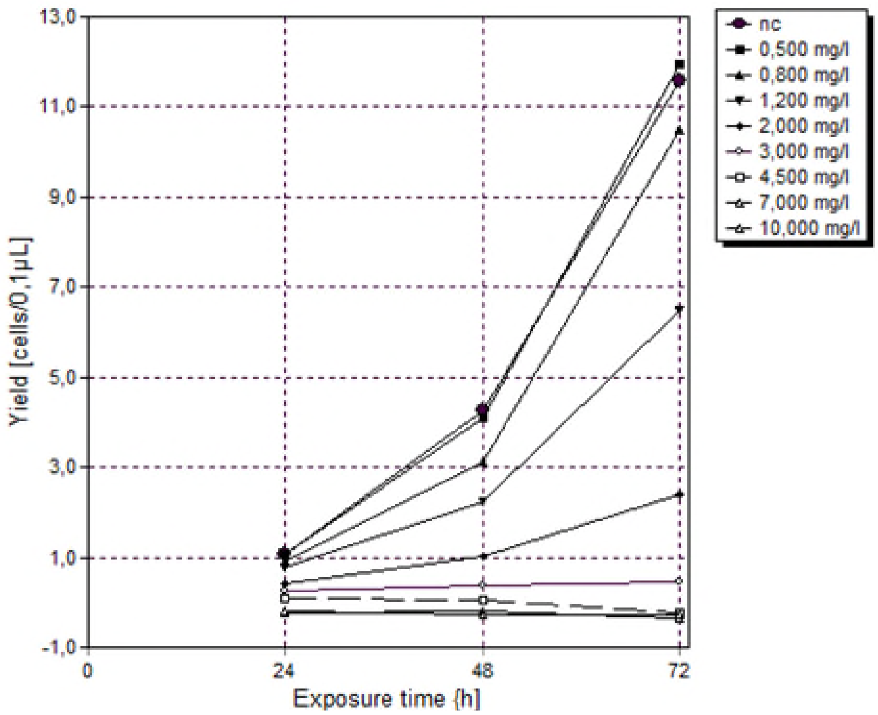
Figure 3: Cell concentration of *P. tricornutum* in relation to test item 3,5-DCP concentration and exposure time



Source: own illustration, German Environment Agency

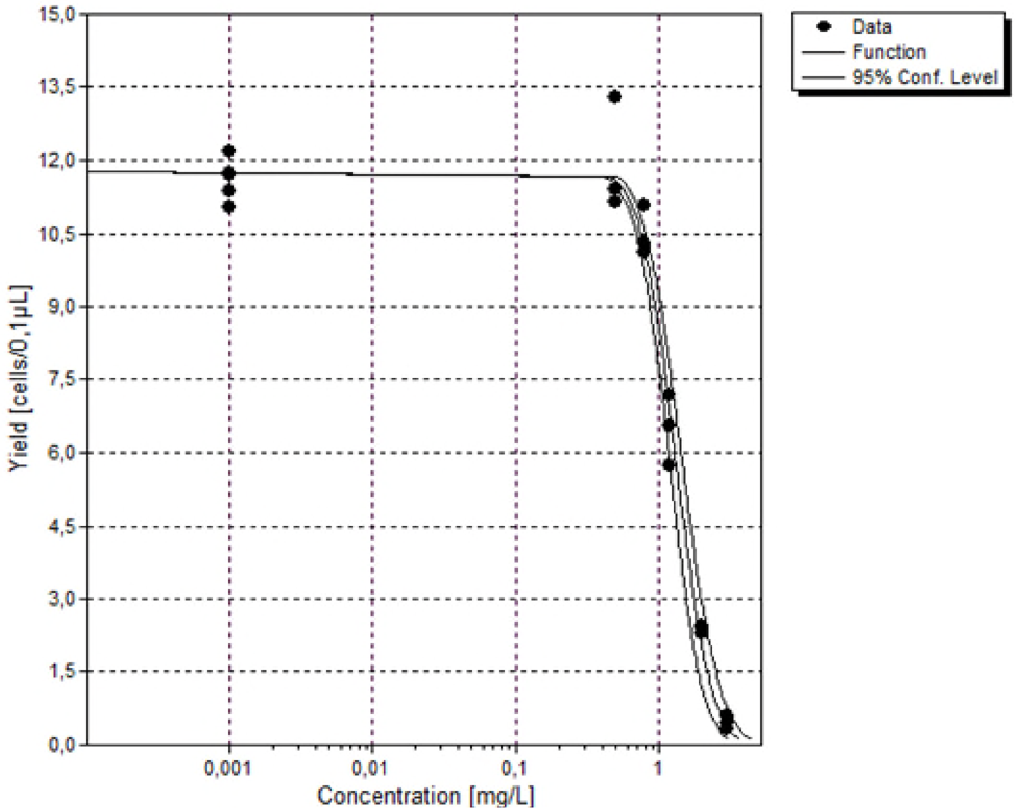
Toxicological endpoint: Yield

Figure 4: Yield of *P. tricornutum* and test item 3,5-DCP over exposure time



Source: own illustration, German Environment Agency

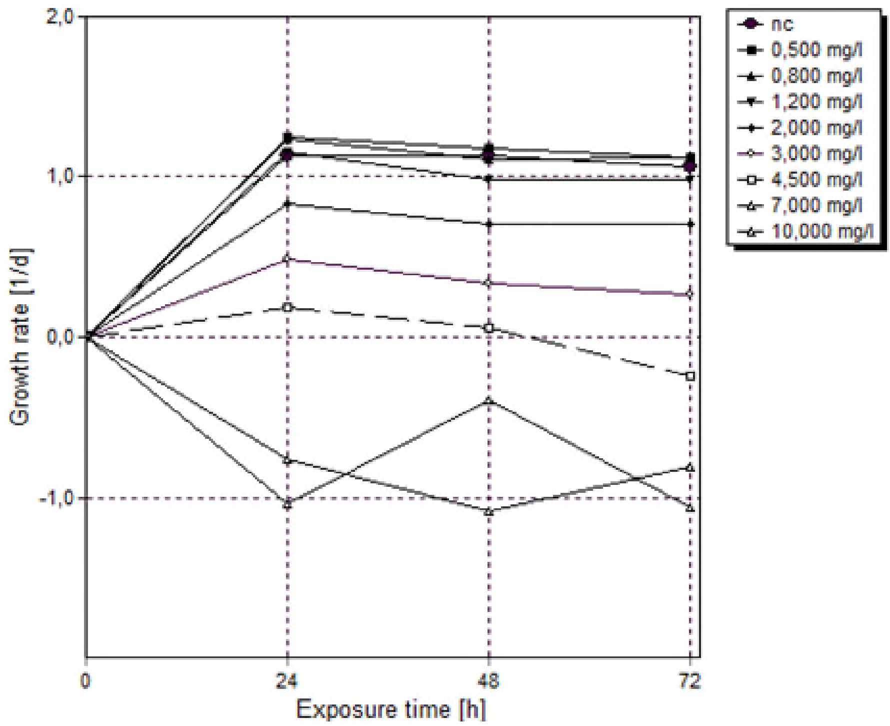
Figure 5: Yield of *P. tricornutum* over test item 3,5-DCP concentration



Source: own illustration, German Environment Agency

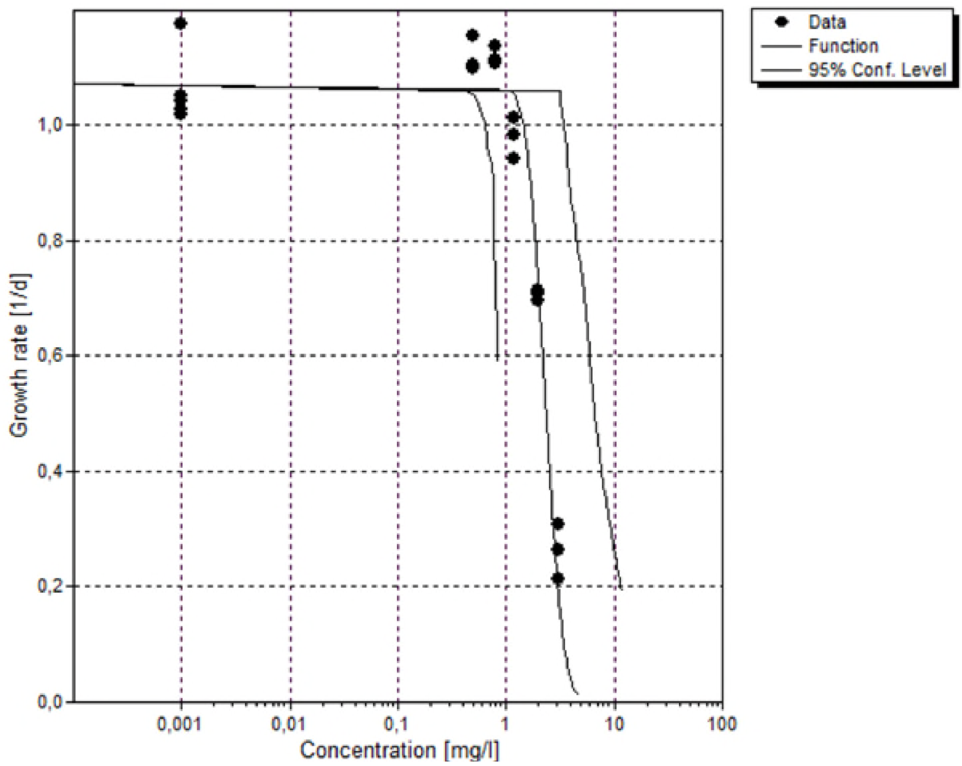
Toxological endpoint: <u>Growth rate</u>

Figure 6: Growth rate of *P. tricornutum* and test item 3,5-DCP over exposure time



Source: own illustration, German Environment Agency

Figure 7: Growth rate of *P. tricornutum* over test item 3,5-DCP concentration



Source: own illustration, German Environment Agency

3.2.2 *Skeletonema marinoi*

Schelzig, S. (2022b): Prüfung der Toxizität von 3,5-Dichlorphenol auf marine Kieselalgen der Art *Skeletonema marinoi* (Referenztest no. C04Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C04Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124181?sv=

The results of the reference testing of the toxicity of 3,5 DCP on *Skeletonema marinoi* are shown in the tables 13 and 14 and figures 8, 9, 10, 11 and 12.

Effect concentrations

Table 13: Effect concentration for test item 3,5-DCP

EC_x [mg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	0.50	1.187
lower CL	0.494	< 0.001
upper CL	0.515	n.d.[5]
EC_{50}	0.989	1.254
lower CL	0.964	n.d.
upper CL	1.013	n.d.
LOEC	≤ 0.300	1.200
NOEC	< 0.300	0.800

[5] Not determined due to mathematical reasons or inappropriate data

Table 14 shows the inhibition of yield and growth rate over 72 hours by test item 3,5-DCP

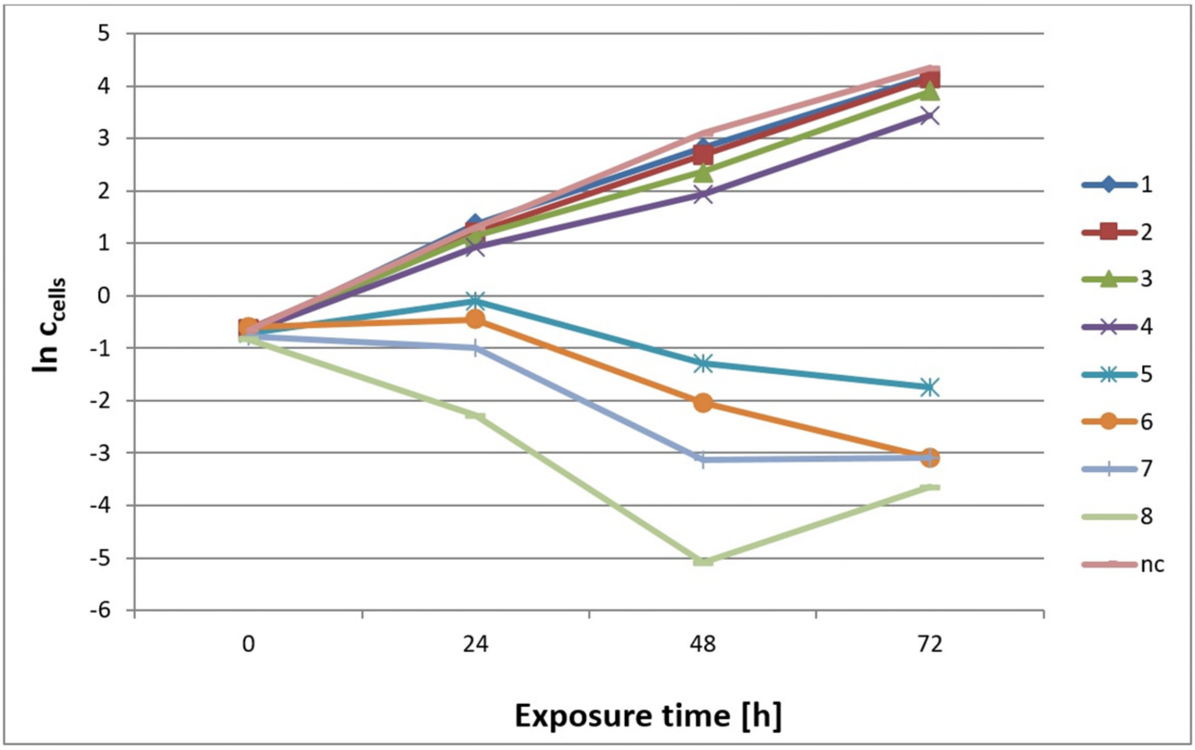
Table 14: Inhibition of yield and growth rate over 72 hours by test item 3,5-DCP

sample	test item concentration[6] [mg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	0.3	14.7	2.6
2	0.5	18.7	4.4
3	0.8	36.5	8.7
4	1.2	60.6	17.8
5	2.0	100.4	121.7
6	3.0	100.7	152.2
7	4.5	100.5	148.5
8	7.0	100.5	156.5

Dose-response relationship

Cell concentration over exposure time

Figure 8: Cell concentration of *S. marinoi* in relation to test item 3,5-DCP concentration and exposure time

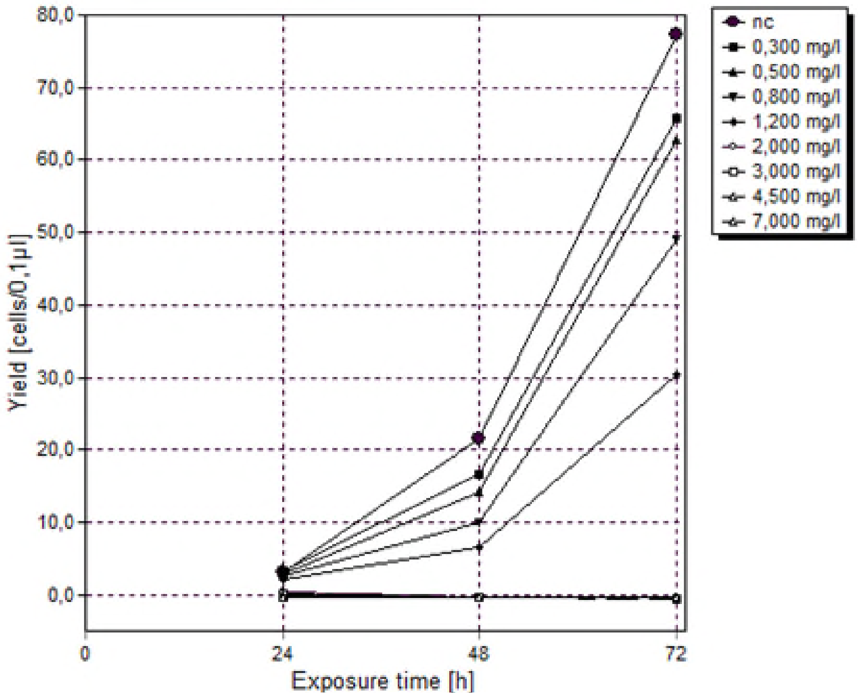


Source: own illustration, German Environment Agency

[6] nominal

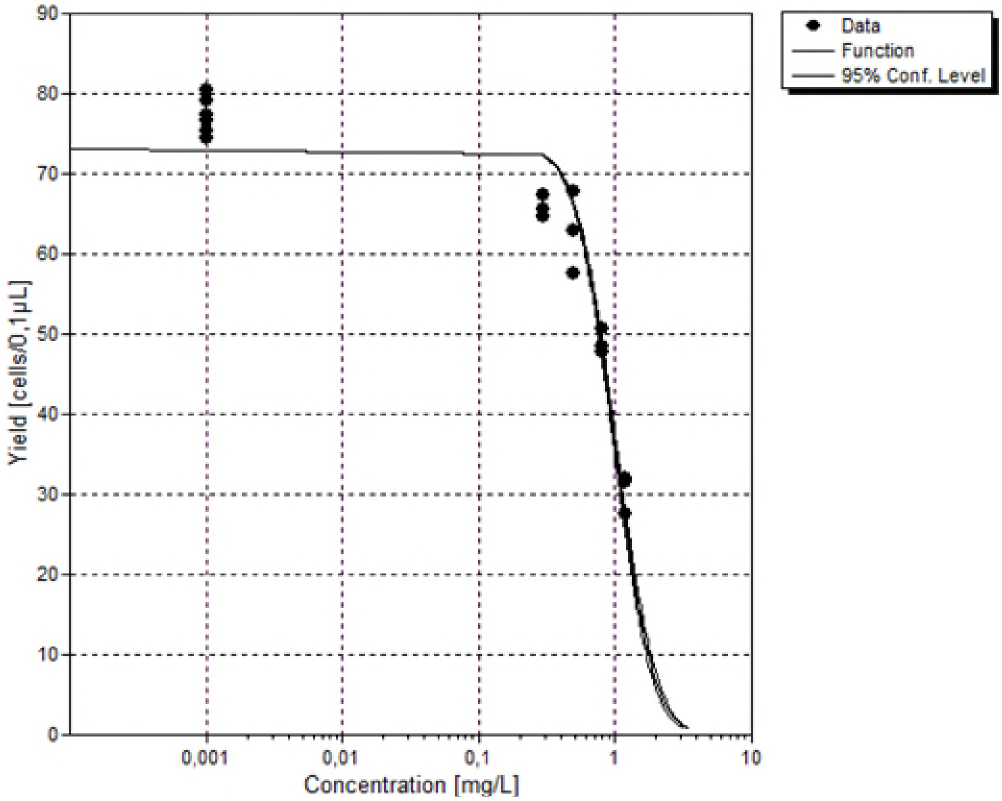
Toxicological endpoint: <u>Yield</u>

Figure 9: Yield of *S. marinoi* and test item 3,5-DCP over exposure time



Source: own illustration, German Environment Agency

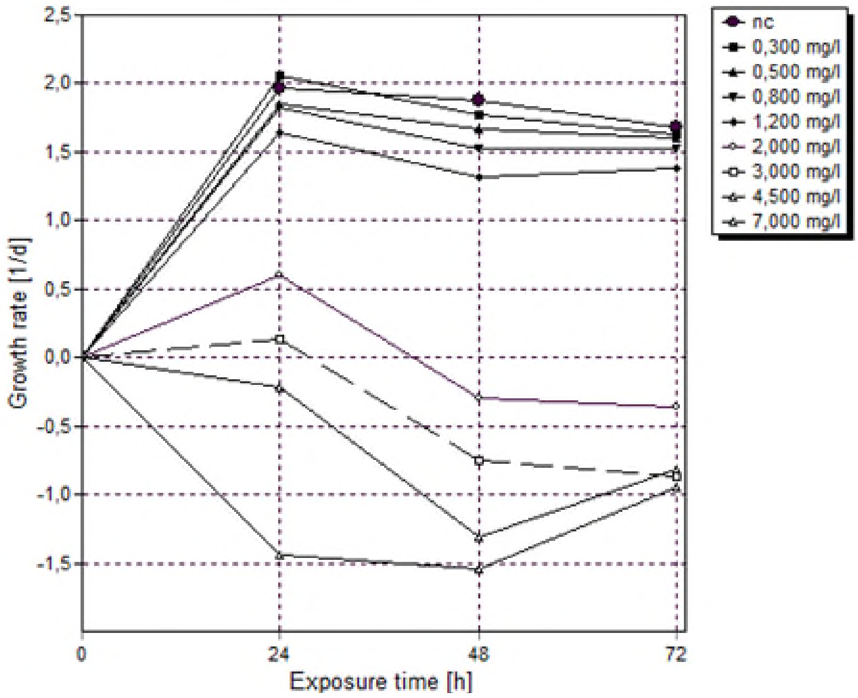
Figure 10: Yield of *S. marinoi* over test item 3,5-DCP concentration



Source: own illustration, German Environment Agency

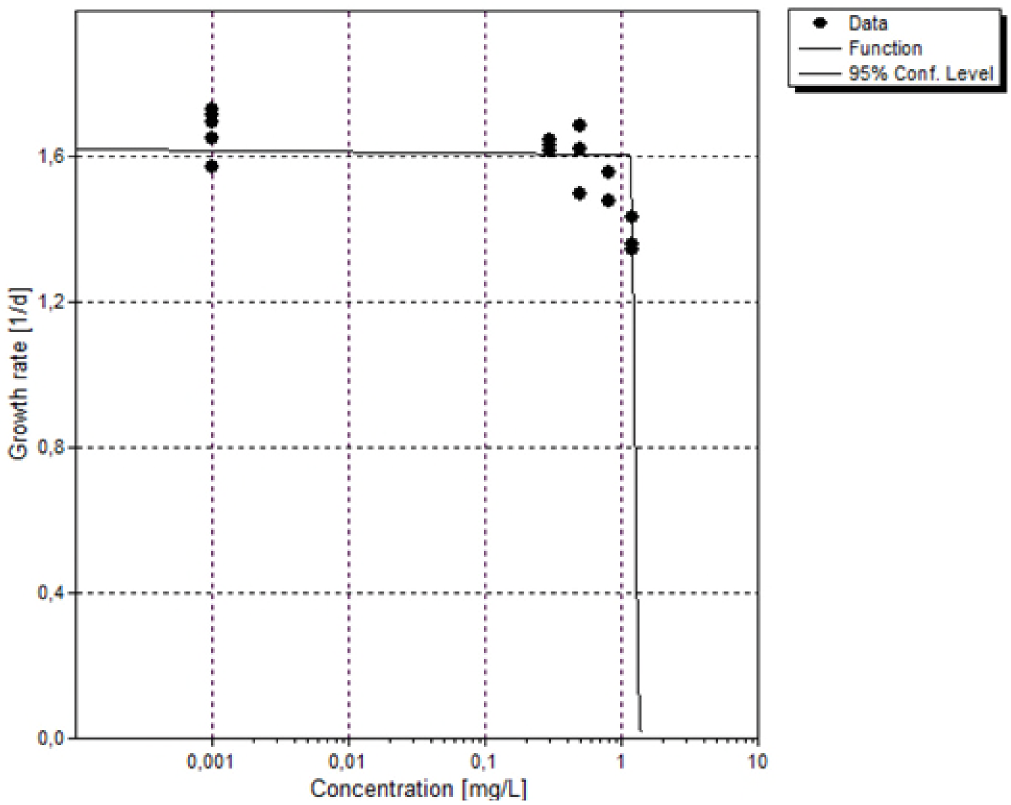
Toxicological endpoint: <u>Growth rate</u>

Figure 11: Growth rate of *S. marinoi* and test item 3,5-DCP over exposure time



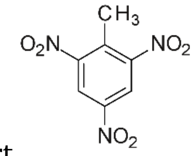
Source: own illustration, German Environment Agency

Figure 12: Growth rate of *S. marinoi* over test item 3,5-DCP concentration



Source: own illustration, German Environment Agency

3.3 TNT (2,4,6-Trinitrotoluol)



3.3.1 *Phaeodactylum tricornutum*

Schelzig, S. (2022c): Prüfung der Toxizität von TNT auf marine Kieselalgen der Art *Phaeodactylum tricornutum* (Test no. C07Pt). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C07Pt.

https://recherche.chemikalieninfo.de/etox/zitat/122650?sv=

The results of the testing of the toxicity of TNT on *Phaeodactylum tricornutum* are shown in the tables 15, 16 and 17 and figures 13, 14, 15, 16 and 17.

Analytical determination of test item concentration

Table 15: Analytical determination of test item TNT

sample	nominal concentration [µg/L]	true concentration[7] [µg/L]	recovery rate [%]
nc	0	0	-
1	0.5	0.275	55.0
2	1.3	0.61	47.3
3	4.0	1.160	29.0
4	10.0	2.659	26.6
5	25.0	9.310	37.2
6	70.0	35.322	50.5
7	200.0	153.54	76.8
8	500.0	262.041	52.4
9	1500.0	1412.014	94.1
10	3500.0	3935.060	112.4

[7] Geometric mean of test item concentration over the exposure time of 72 hours

Effect concentrations

Table 16: Effect concentrations for test item TNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	41.751	1320.650
lower CL	16.477	< 0.001
upper CL	105.793	n.d.
EC_{50}	412.280	1468.173
lower CL	130.899	n.d.
upper CL	1252.042	n.d.
LOEC	2.659	≤ 0.275
NOEC	1.160	≤ 0.275

Inhibition of yield and growth rate

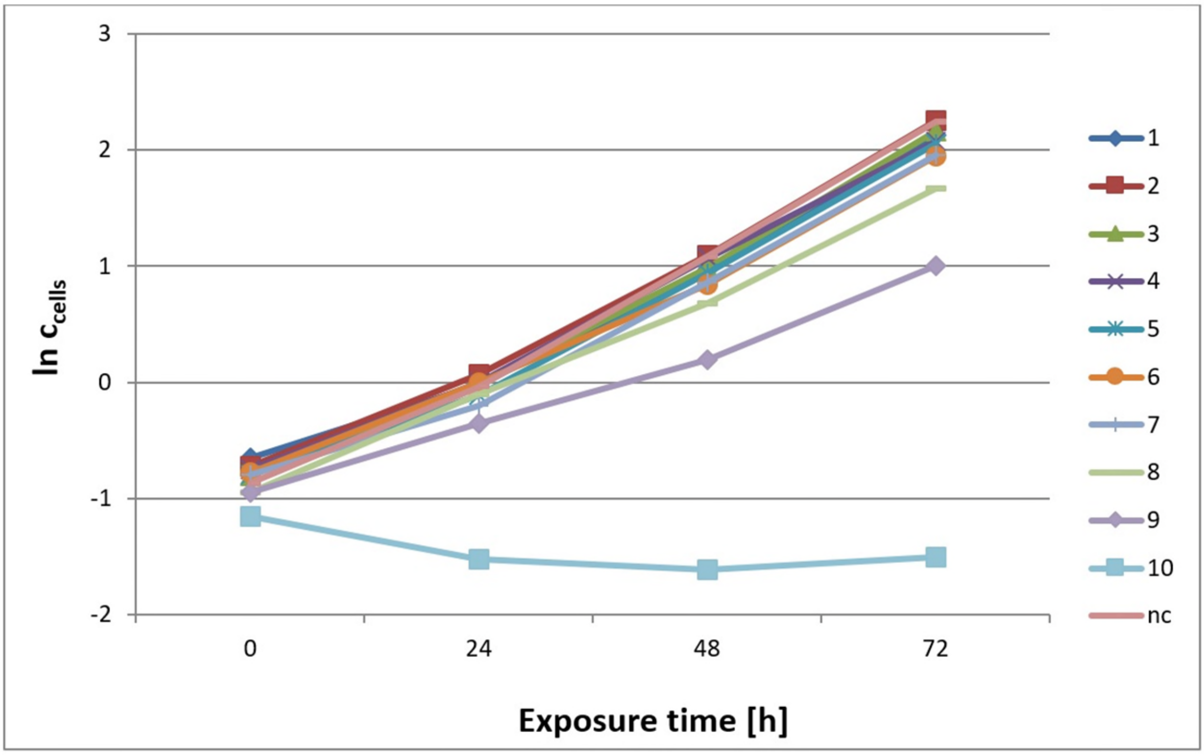
Table 17: Inhibition of yield and growth rate over 72 hours by test item TNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	0.275	12.7	10.7
2	0.61	-0.8	4.2
3	1.160	7.8	4.2
4	2.659	17.1	9.3
5	9.310	18.4	8.2
6	35.322	26.9	12.3
7	153.545	26.0	11.4
8	262.041	45.1	15.8
9	1412.014	74.0	37.0
10	3935.060	101.0	111.4

Dose-response relationships

Cell concentration over exposure time

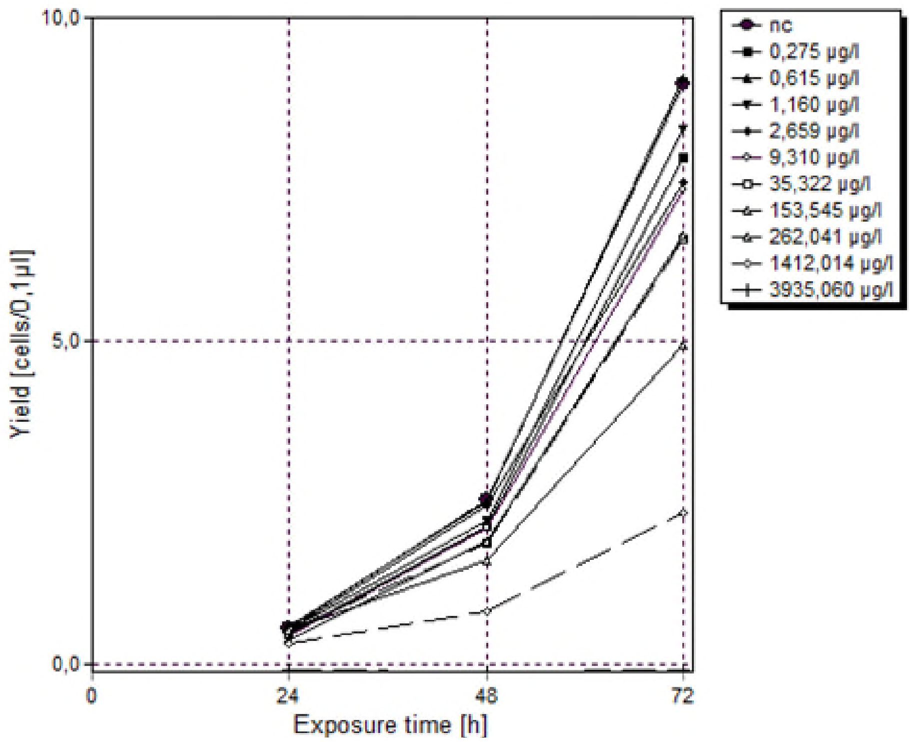
Figure 13: Cell concentration of *P. tricornutum* in relation to test item TNT concentration and exposure over time



Source: own illustration, German Environment Agency

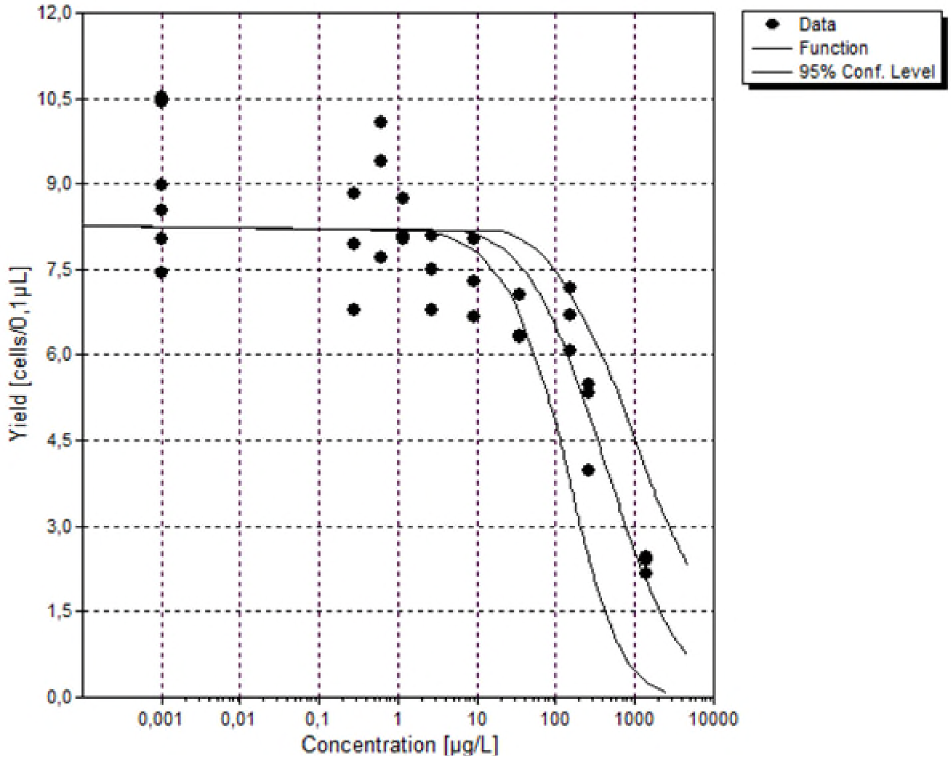
Toxical endpoint: Yield

Figure 14: Yield of *P. tricornutum* and test item TNT over exposure time



Source: own illustration, German Environment Agency

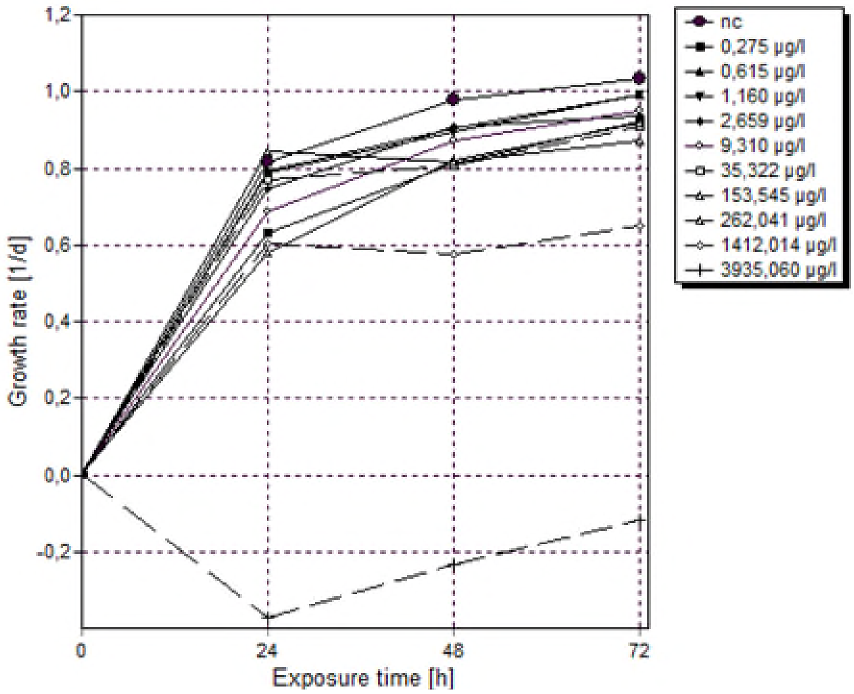
Figure 15: Yield of *P. tricornutum* over test item TNT concentration



Source: own illustration, German Environment Agency

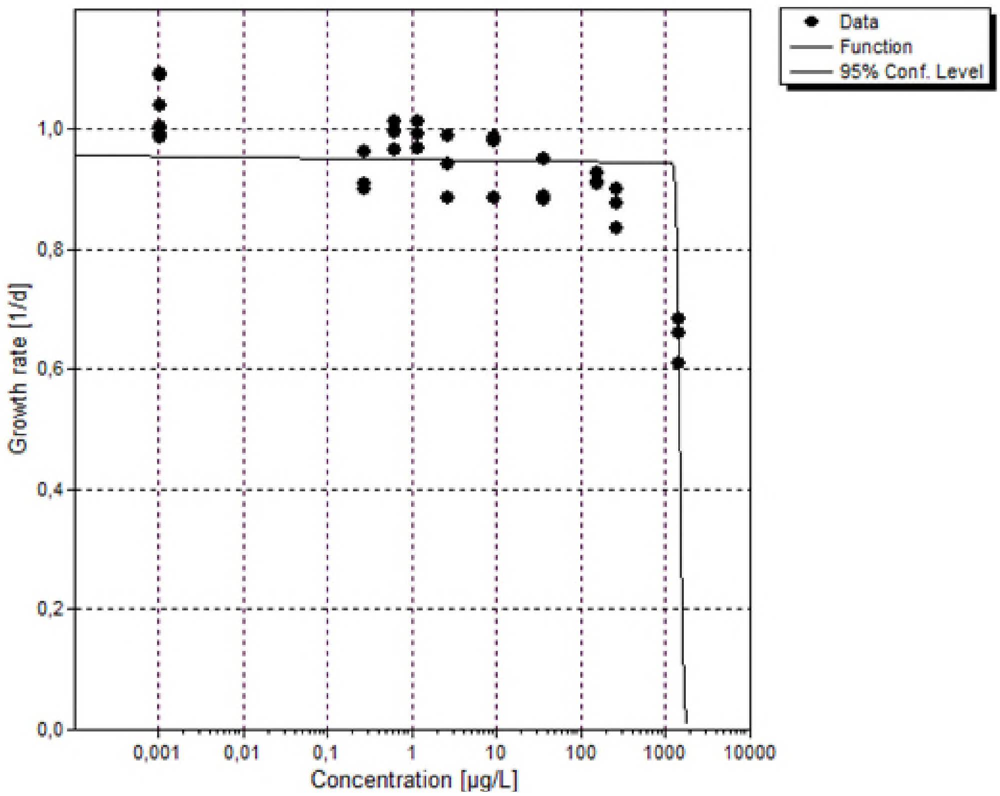
Toxicological endpoint: Growth rate

Figure 16: Growth rate of *P. tricornutum* and test item TNT over exposure time



Source: own illustration, German Environment Agency

Figure 17: Growth rate of *P. tricornutum* over test item TNT concentration



Source: own illustration, German Environment Agency

3.3.2 *Skeletonema marinoi*

Schelzig, S. (2023a): Prüfung der Toxizität von TNT auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no. C13Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C13Sm.

https://recherche.chemikalieninfo.de/etox/zitat/122651?sv=

The results of the testing of the toxicity of TNT on *Skeletonema marinoi* are shown in the tables 18, 19 and 20 and figures 18, 19, 20, 21 and 22.

Analytical determination of test item concentration

Table 18: Analytical determination of test item TNT

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	125.0	80.585	64.5
2	150.0	107.582	71.7
3	175.0	115.413	66.0
4	200.0	137.699	68.8
5	225.0	158.507	70.4
6	250.0	192.206	76.9
7	300.0	206.595	68.9

Effect concentration

Table 19: Effect concentrations for test item TNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	61.818	130.022
lower CL	61.613	122.238
upper CL	62.025	138.301
EC_{50}	108.312	181.771
lower CL	107.889	168.185
upper CL	108.741	195.938
LOEC	≤ 80.585	> 206.595
NOEC	< 80.585	≥ 206.595

Inhibition of yield and growth rate

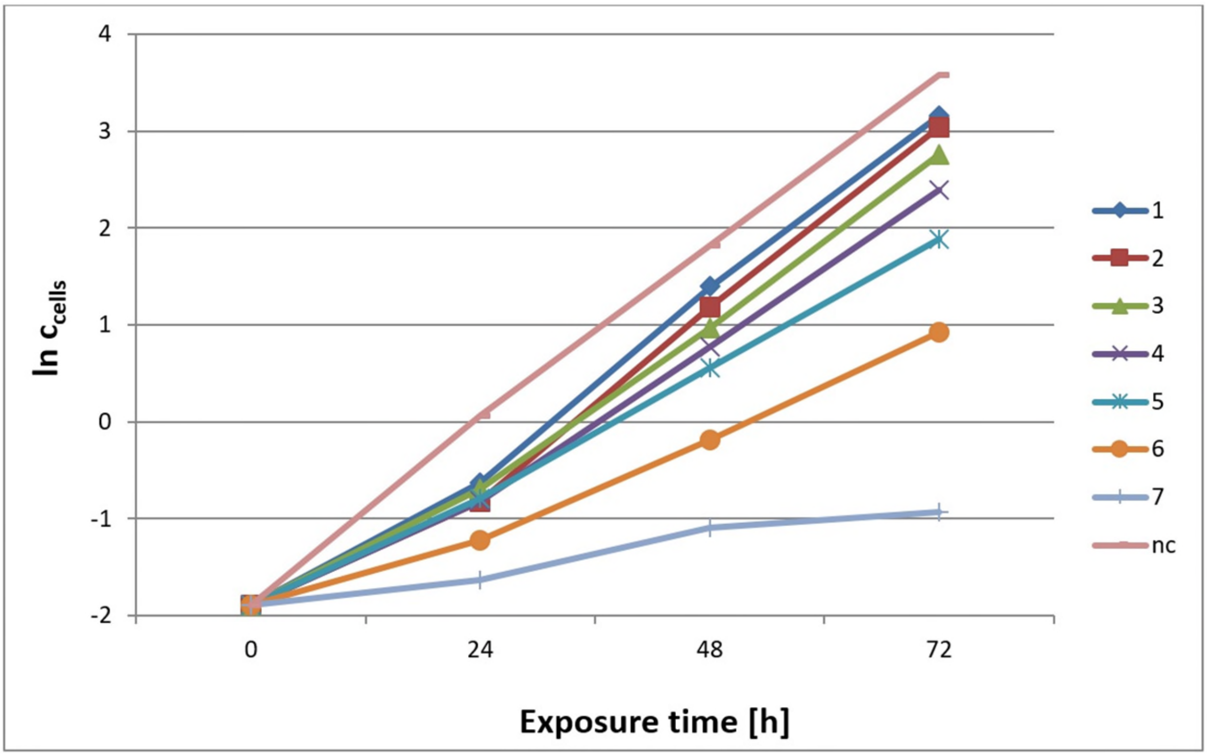
Table 20: Inhibition of yield and growth rate over 72 hours by test item TNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	80.585	34.8	7.8
2	107.582	42.2	10.0
3	115.413	56.5	15.1
4	137.699	69.9	21.9
5	158.507	82.0	31.1
6	192.206	93.4	49.1
7	206.595	99.3	83.0

Dose-response relationships

Cell concentration over exposure time

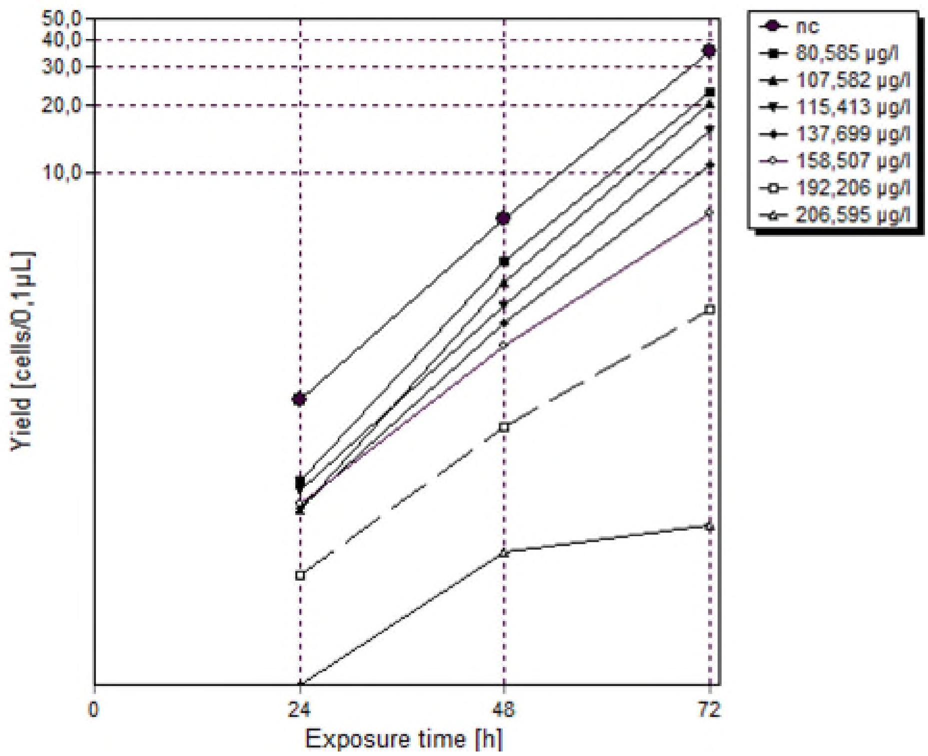
Figure 18: Cell concentration of *S. marinoi* in relation to test item TNT concentration and exposure time



Source: own illustration, German Environment Agency

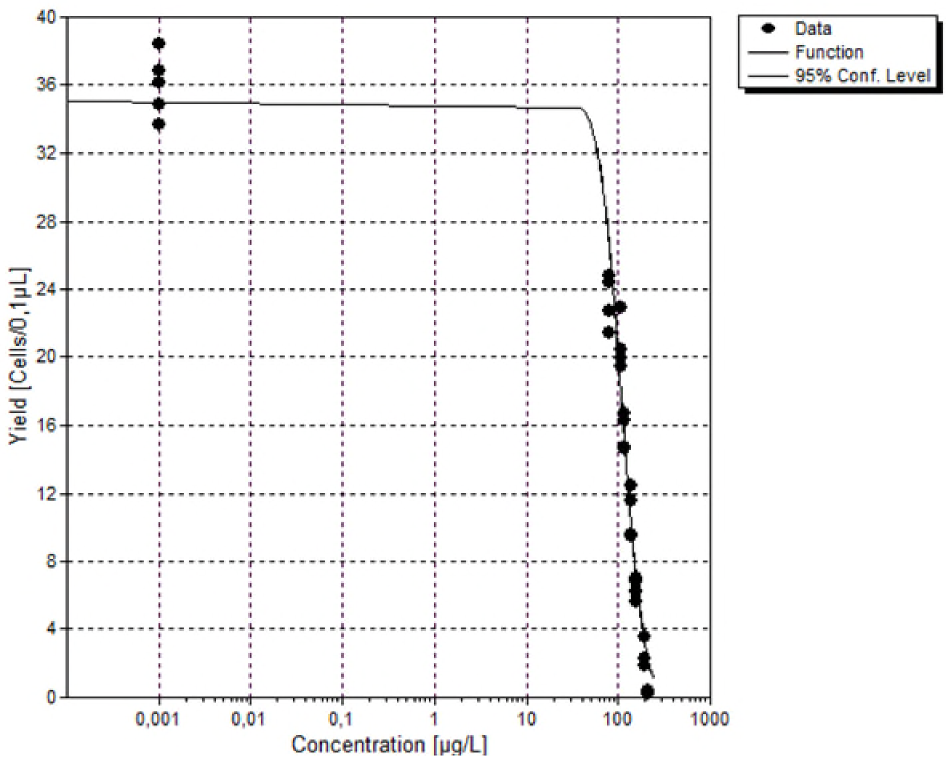
Toxicological endpoint: <u>Yield</u>

Figure 19: Yield of *S. marinoi* and test item TNT over exposure time



Source: own illustration, German Environment Agency

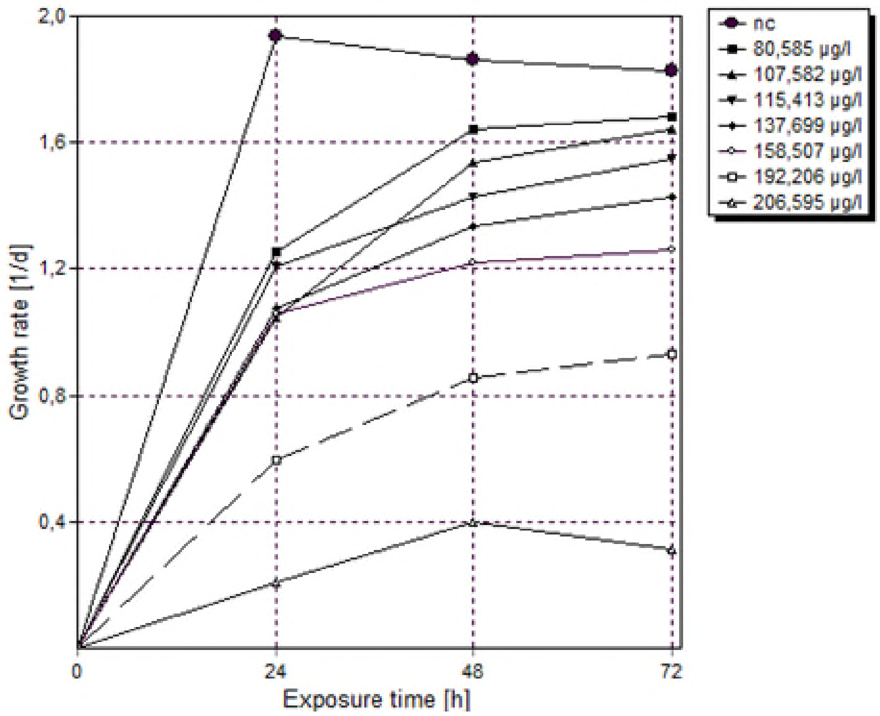
Figure 20: Yield of *S. marinoi* over test item TNT concentration



Source: own illustration, German Environment Agency

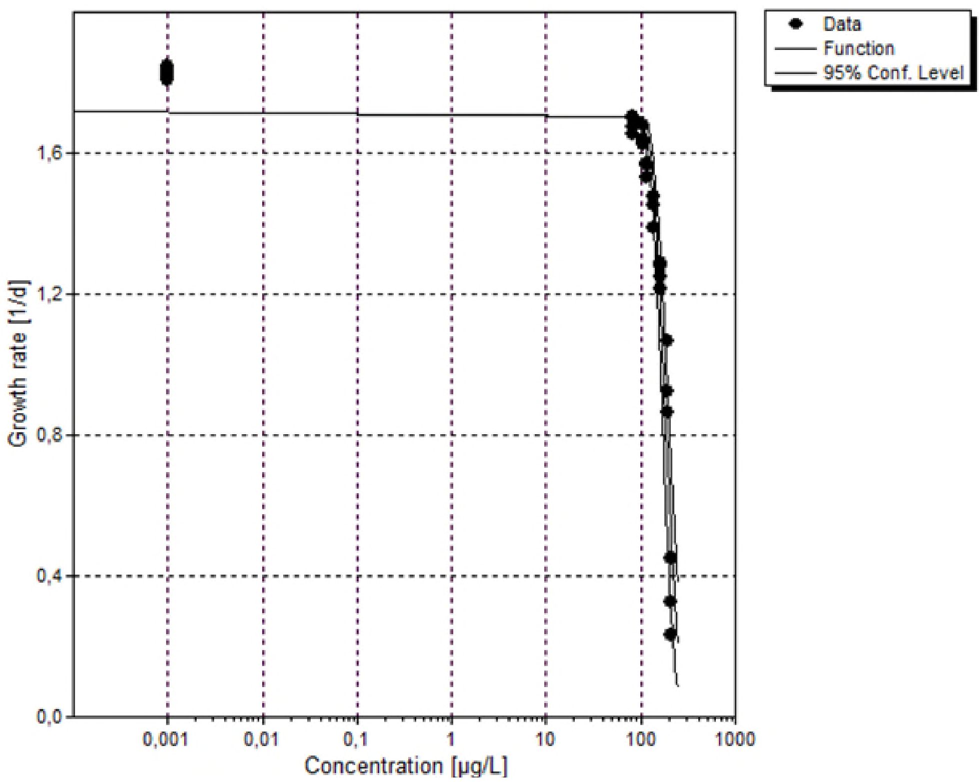
Toxicological endpoint: Growth rate

Figure 21: Growth rate of *S. marinoi* and test item TNT over exposure time



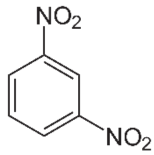
Source: own illustration, German Environment Agency

Figure 22: Growth rate of *S. marinoi* over test item TNT concentration



Source: own illustration, German Environment Agency

3.4 1,3-DNB (1,3-Dinitrobenzene)



3.4.1 Skeletonema marinoi

Schelzig, S. (2023b): Prüfung der Toxizität von 1,3-Dinitrobenzol auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no. C17Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C17Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124176?sv=

The results of the testing of the toxicity of 1,3-DNB on *Skeletonema marinoi* are shown in the tables 21, 22 and 23 and figures 23, 24, 25, 26 and 27.

Analytical determination of test item concentration

Table 21: Analytical determination of test item 1,3-DNB

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	150.0	123.6	82.4
2	300.0	288.0	96.0
3	450.0	463.91	103.1
4	600.0	519.72	86.6
5	750.0	650.48	86.7
6	900.0	782.13	86.9
7	1050.0	936.18	89.2

Effect concentration

Table 22: Effect concentration for test item 1,3-DNB

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	461.615	634.413
lower CL	438.959	< 0.001
upper CL	485.441	n.d.
EC_{50}	533.826	649.349
lower CL	498.028	< 0.001
upper CL	569.503	n.d.
LOEC	519.723	463.913
NOEC	463.913	288.071

Inhibition of yield and growth rate

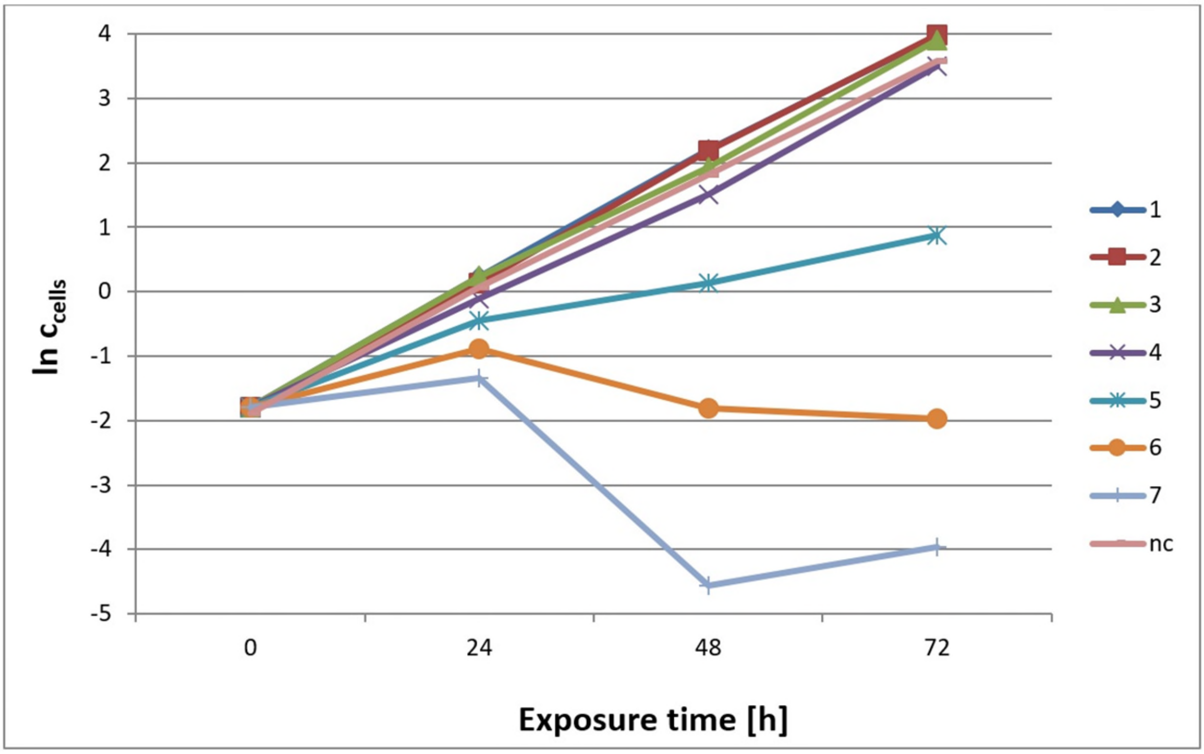
Table 23: Inhibition of yield and growth rate over 72 hours by test item 1,3-DNB

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	123.65	10.8	2.0
2	288.07	8.5	1.5
3	463.91	15.8	3.1
4	519.72	43.9	9.9
5	650.48	96.2	55.2
6	782.13	100.0	103.5
7	936.18	100.2	118.1

Dose-response relationships

Cell concentration over exposure time

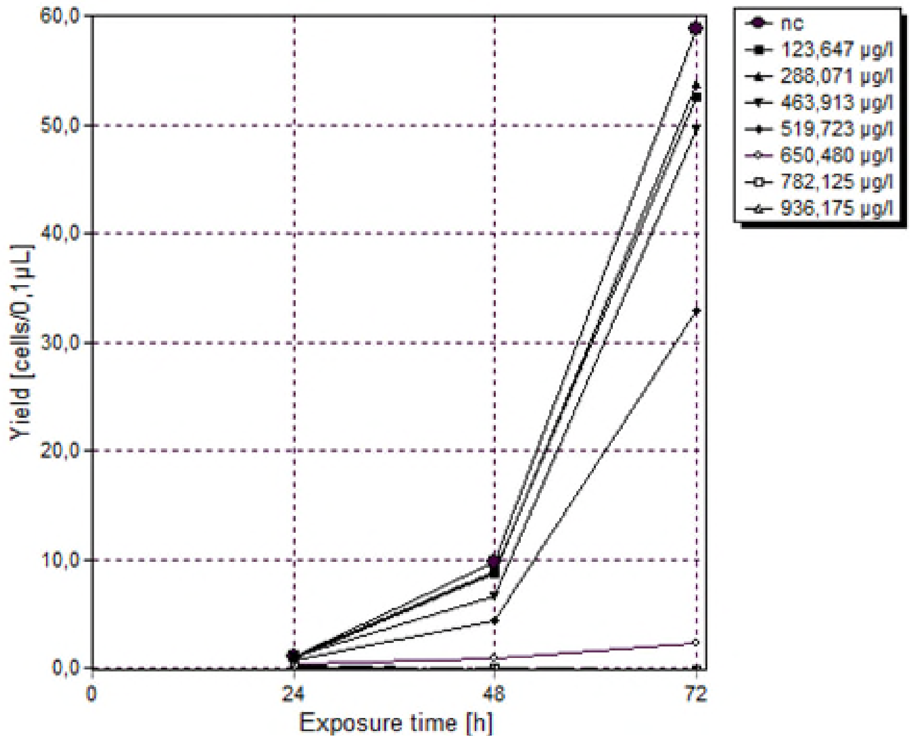
Figure 23: Cell concentration of *S. marinoi* in relation to test item 1,3-DNB concentration and exposure time



Source: own illustration, German Environment Agency

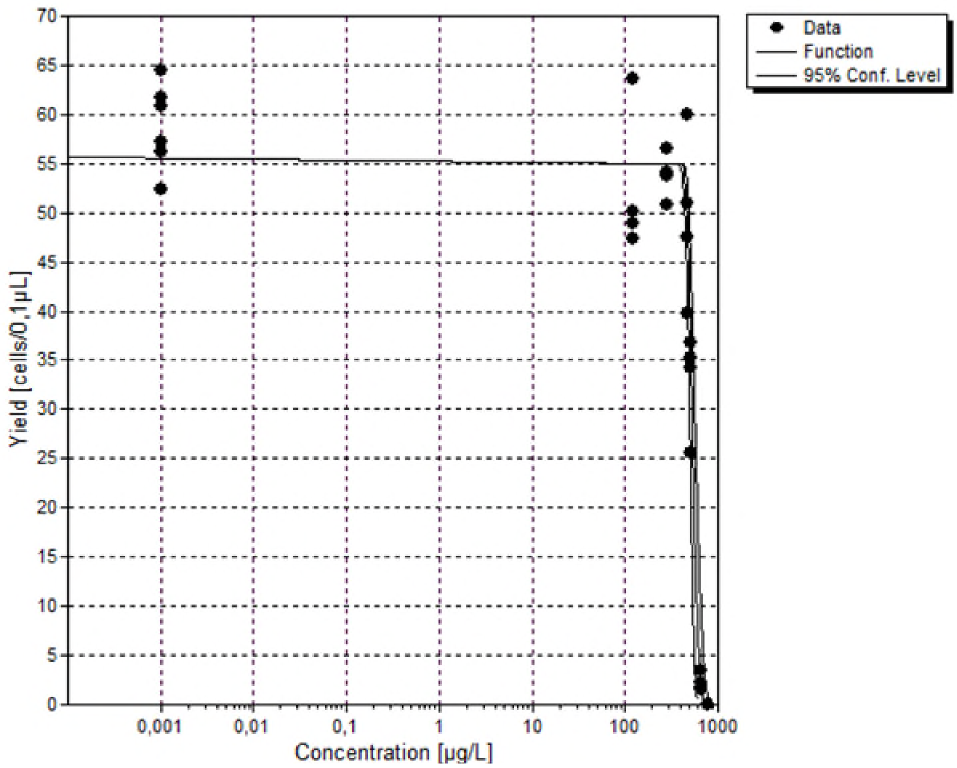
Toxicological endpoint: Yield

Figure 24: Yield of *S. marinoi* and test item 1,3-DNB over exposure time



Source: own illustration, German Environment Agency

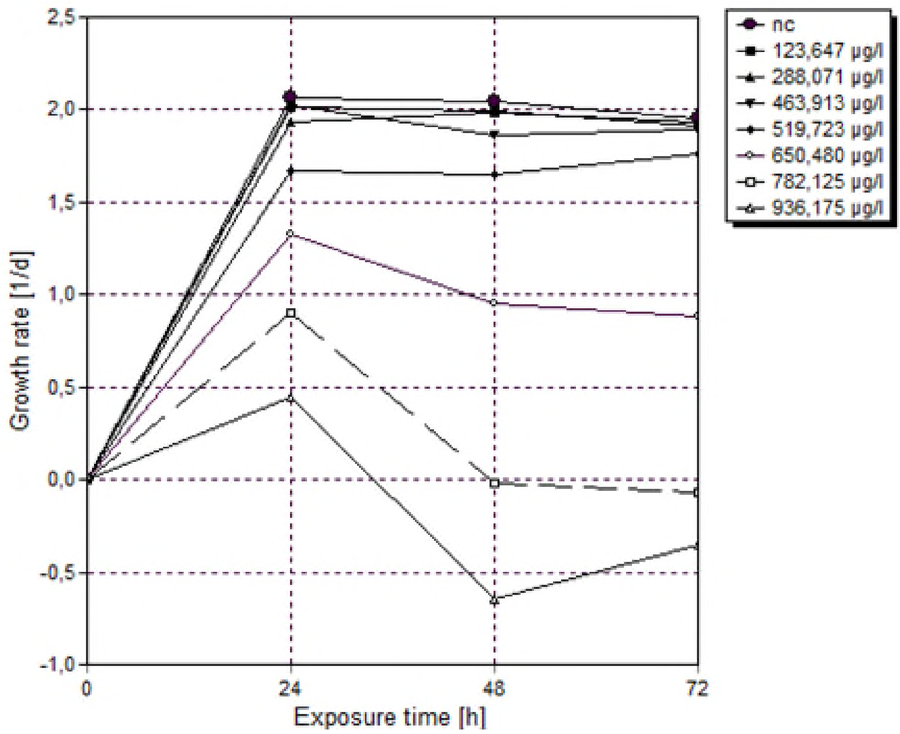
Figure 25: Yield of *S. marinoi* over test item 1,3-DNB concentration



Source: own illustration, German Environment Agency

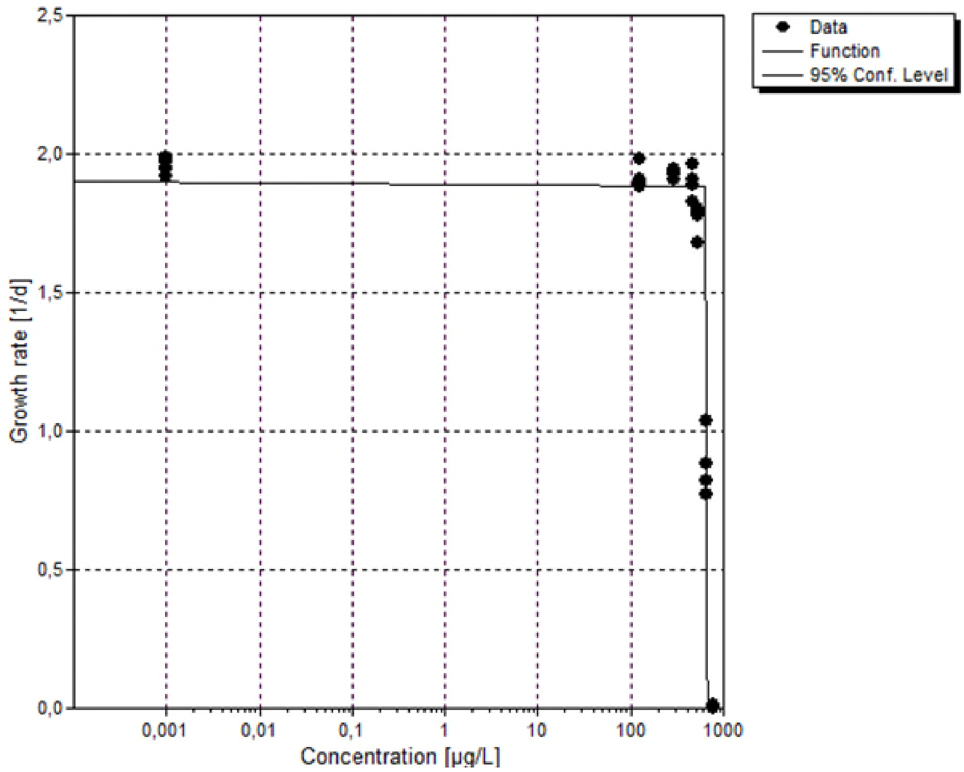
Toxicological endpoint: Growth rate

Figure 26: Growth rate of *S. marinoi* and test item 1,3-DNB over exposure time



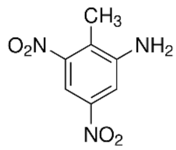
Source: own illustration, German Environment Agency

Figure 27: Growth rate of *S. marinoi* over test item 1,3-DNB concentration



Source: own illustration, German Environment Agency

3.5 2-ADNT (2-Amino-4,6-dinitrotoluene)



3.5.1 *Skeletonema marinoi*

Schelzig, S.: (2023c) Prüfung der Toxizität von 2-Amino-4,6-dinitrotoluol auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no. C22Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C22Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124177?sv=

The results of the testing of the toxicity of 2-ADNT on *Skeletonema marinoi* are shown in the tables 24, 25 and 26 and figures 28, 29, 30, 31 and 32.

Analytical determination of test item concentration

Table 24: Analytical determination of test item 2-ADNT

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	100.0	21.024	21.02
2	500.0	78.331	15.67
3	900.0	132.760	14.75
4	1200.0	177.459	14.79
5	1500.0	176.050	11.74
6	1800.0	221.136	12.29
7	2100.0	253.710	12.08
8	2800.0	364.737	13.03

Effect concentration

Table 25: Effect concentration for test item 2-ADNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	205.942	230.994
lower CL	197.293	219.899
upper CL	214.970	242.647
EC_{50}	230.217	258.891
lower CL	218.380	240.593
upper CL	242.287	277.551
LOEC	177.459	221.136
NOEC	132.760	176.050

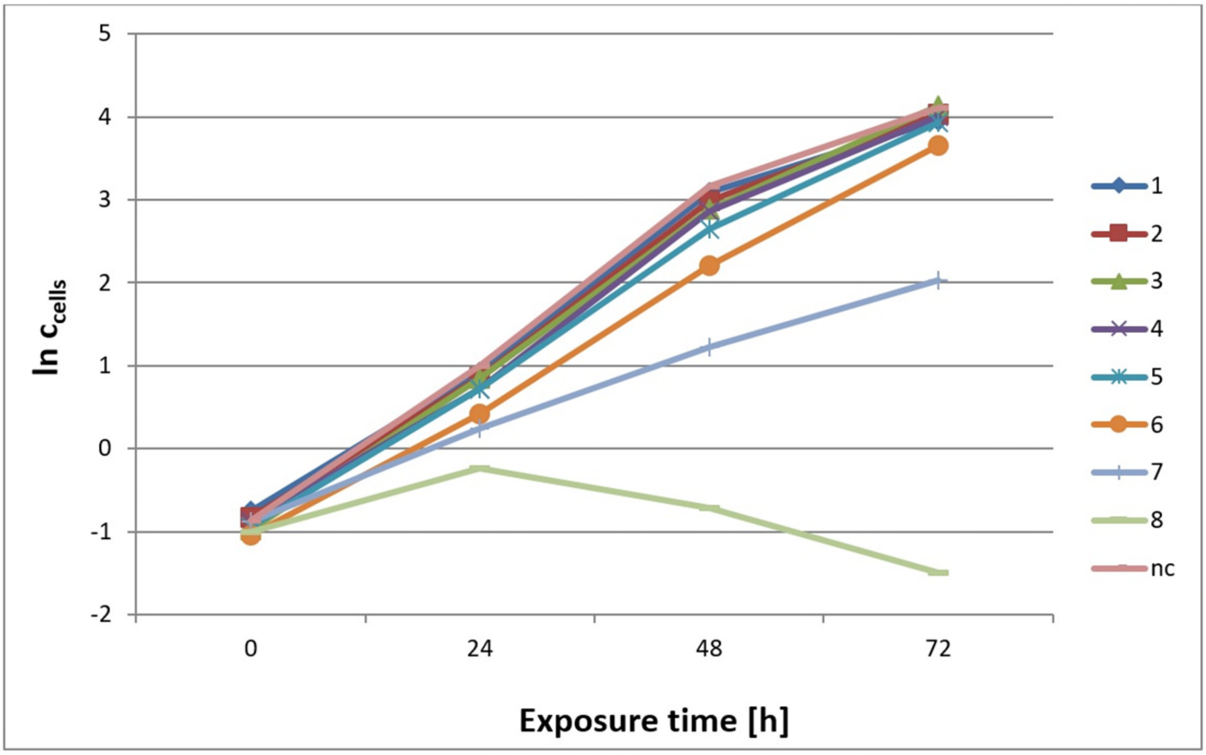
Inhibition of yield and growth rate

Table 26: Inhibition of yield and growth rate over 72 hours by test item 2-ADNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	21.024	14.2	5.5
2	78.331	7.3	2.3
3	132.760	-3.2	-2.8
4	177.459	8.9	1.9
5	176.050	15.4	2.2
6	221.136	35.9	5.2
7	253.710	87.9	41.8
8	364.737	100.2	109.5

Dose-response relationships

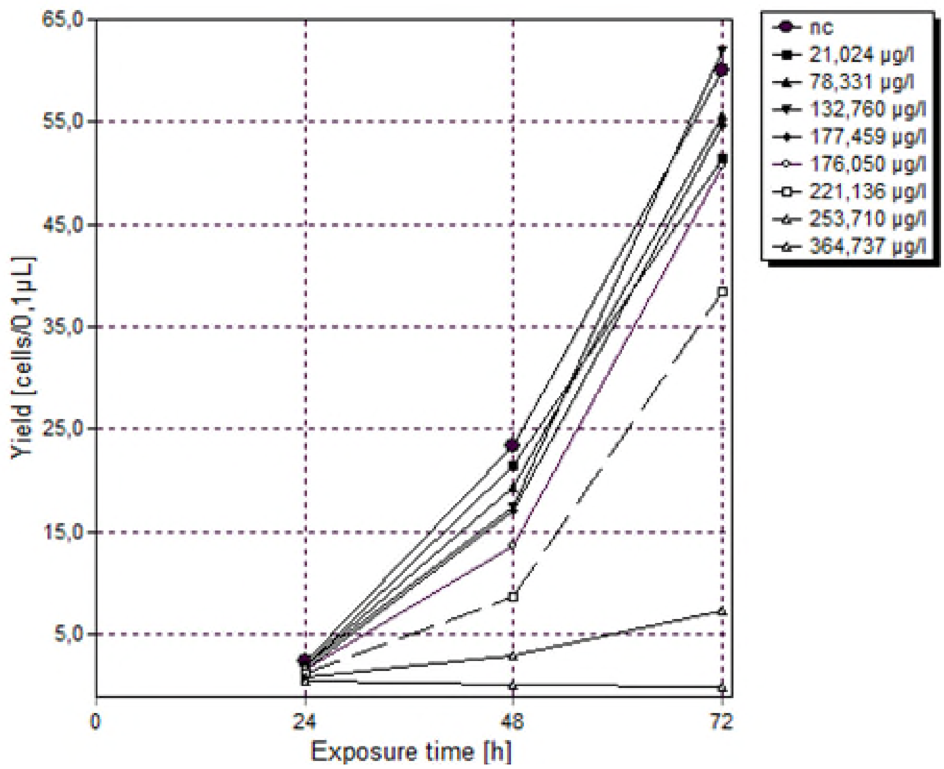
Figure 28: Cell concentration of *S. marinoi* in relation to test item 2-ADNT concentration and exposure time



Source: own illustration, German Environment Agency

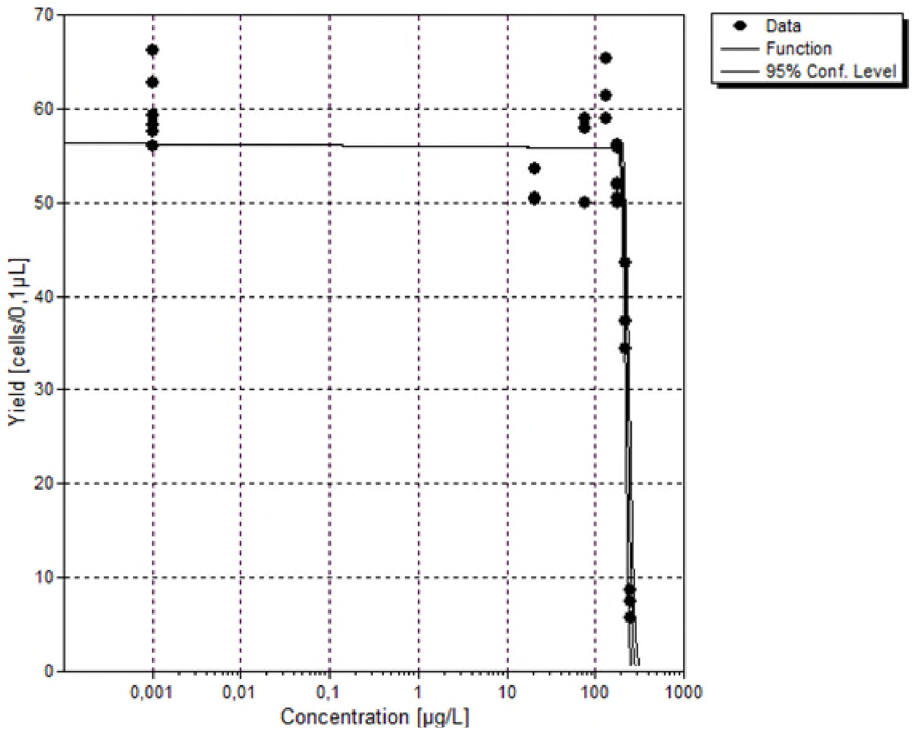
Toxicological endpoint: Yield

Figure 29: Yield of *S. marinoi* and test item 2-ADNT over exposure time



Source: own illustration, German Environment Agency

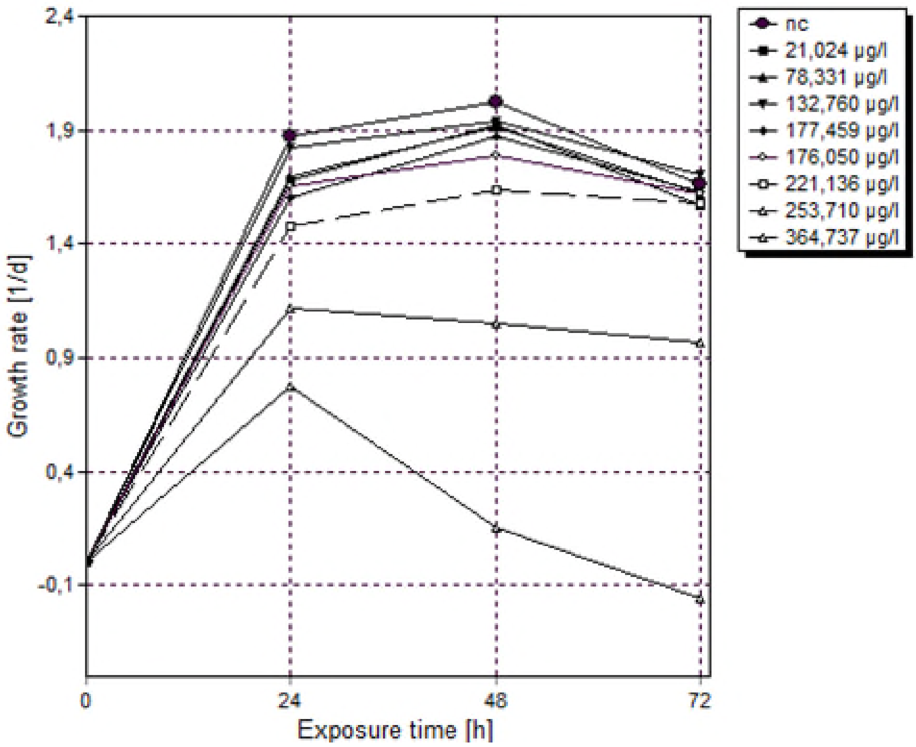
Figure 30: Yield of *S. marinoi* over test item 2-ADNT concentration



Source: own illustration, German Environment Agency

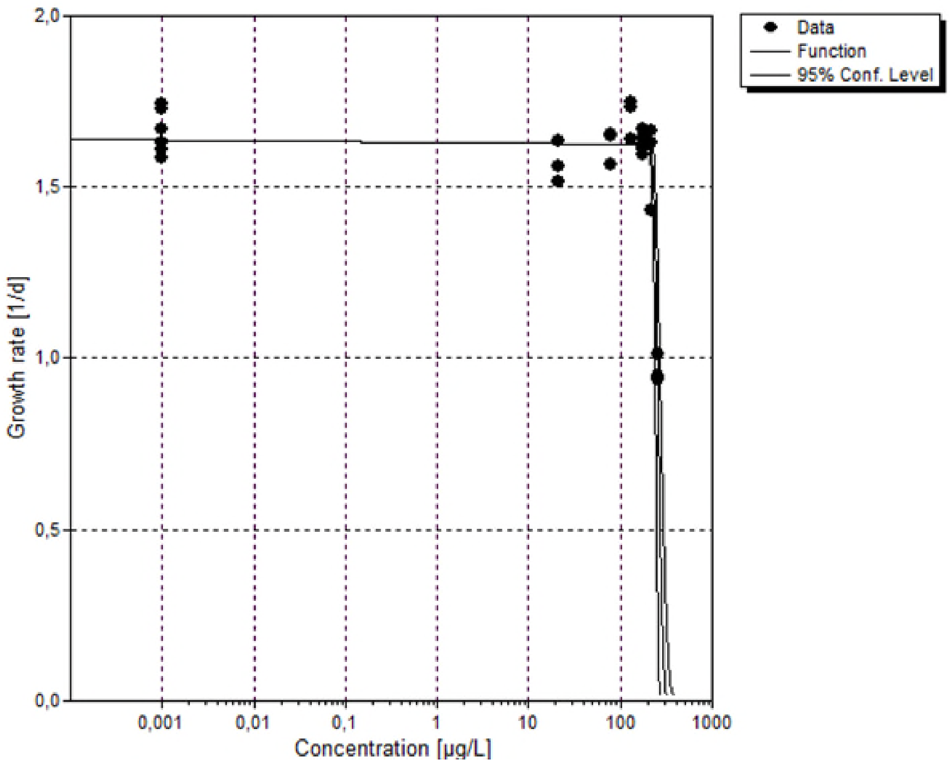
Toxicological endpoint: Growth rate

Figure 31: Growth rate of *S. marinoi* and test item 2-ADNT over exposure time



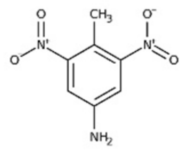
Source: own illustration, German Environment Agency

Figure 32: Growth rate of *S. marinoi* over test item 2-ADNT concentration



Source: own illustration, German Environment Agency

3.6 4-ADNT (4-Amino-2,6-dinitrotoluene)



3.6.1 Skeletonema marinoi

Schelzig, S. (2023d): Prüfung der Toxizität von 4-Amino-2,6-dinitrotoluol auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no. C25Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C25Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124182?sv=

The results of the testing of the toxicity of 4-ADNT on *Skeletonema marinoi* are shown in the tables 27, 28 and 29 and figures 33, 34, 35, 36 and 36 for the first test undertaken.

3.6.1.1 Test 1 of 2

Analytical determinationof test item concentration

Table 27: Analytical determination of test item 4-ADNT

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	500.0	74.016	14.80
2	1000.0	143.116	14.31
3	2000.0	319.455	15.97
4	3000.0	473.451	15.78
5	4000.0	548.448	13.71
6	5000.0	688.190	13.76
7	6000.0	832.116	13.87

Effect concentration

Table 28: Effect concentration for test item 4-ADNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	131.644	236.754
lower CL	123.256	210.250
upper CL	140.602	266.598
EC_{50}	207.476	351.195
lower CL	192.768	306.877
upper CL	223.809	402.814
LOEC	> 832.117	143.116
NOEC	≥ 832.117	74.016

Inhibition of yield and growth rate

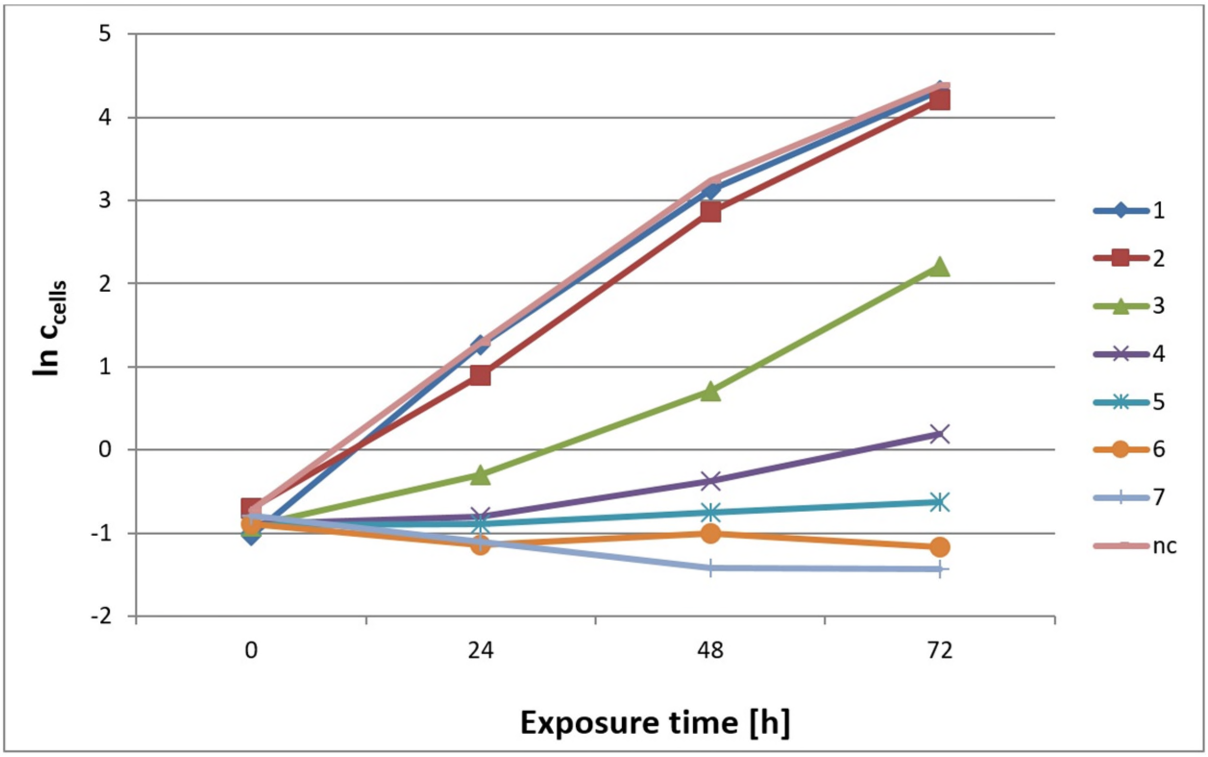
Table 29: Inhibition of yield and growth rate over 72 hours by test item 4-ADNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	74.016	6.1	-4.4
2	143.116	16.6	4.2
3	319.455	89.1	39.2
4	473.451	99.0	78.8
5	548.448	99.8	95.1
6	688.190	100.1	105.6
7	832.116	100.3	112.9

Dose-response relationships

Cell concentration over exposure time

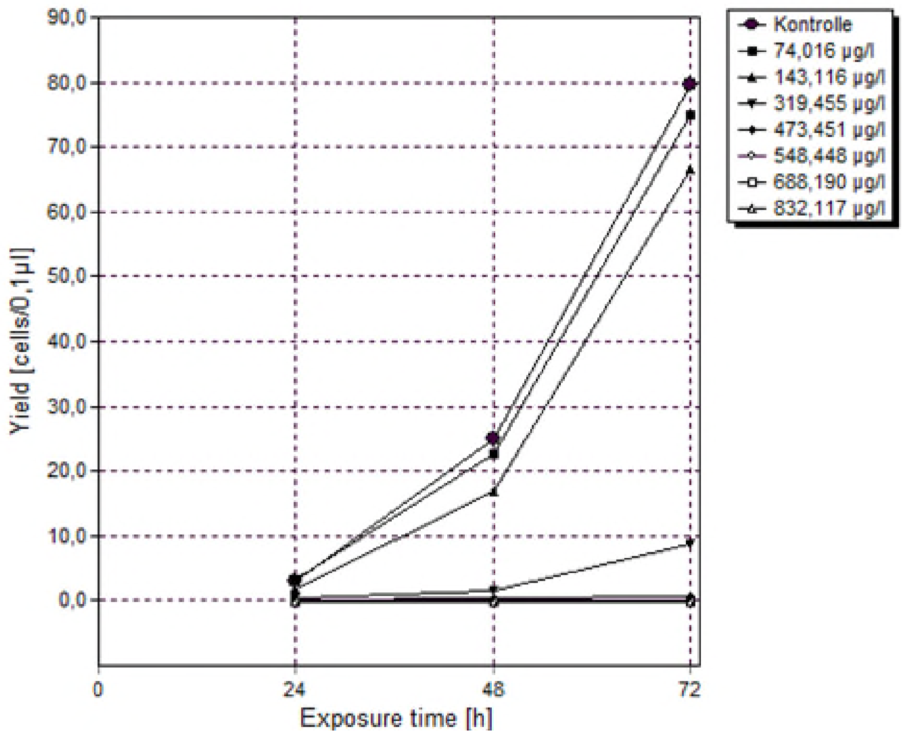
Figure 33: Cell concentration of *S. marinoi* in relation to test item 4-ADNT concentration and exposure time



Source: own illustration, German Environment Agency

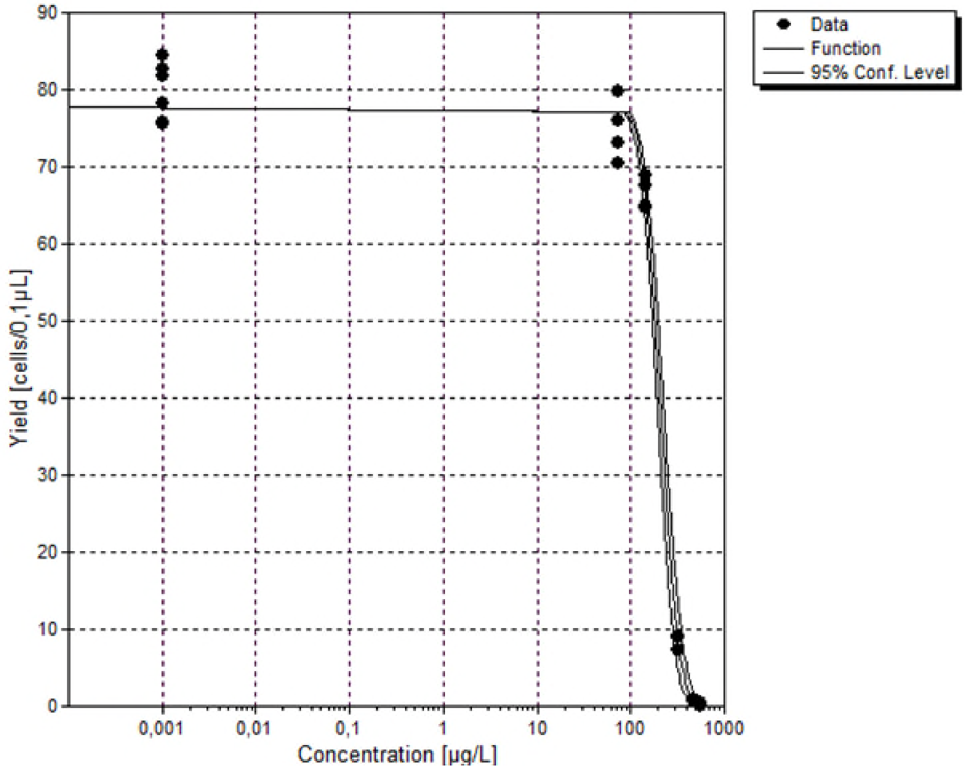
Toxicological endpoint: Yield

Figure 34: Yield of *S. marinoi* and test item 4-ADNT over exposure time



Source: own illustration, German Environment Agency

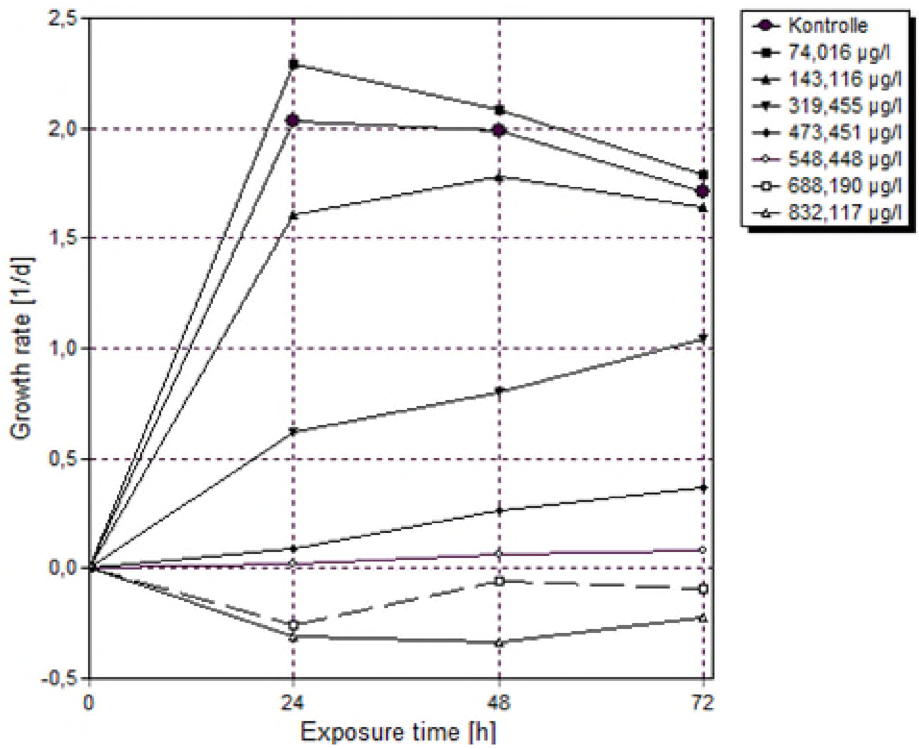
Figure 35: Yield of *S. marinoi* over test item 4-ADNT concentration



Source: own illustration, German Environment Agency

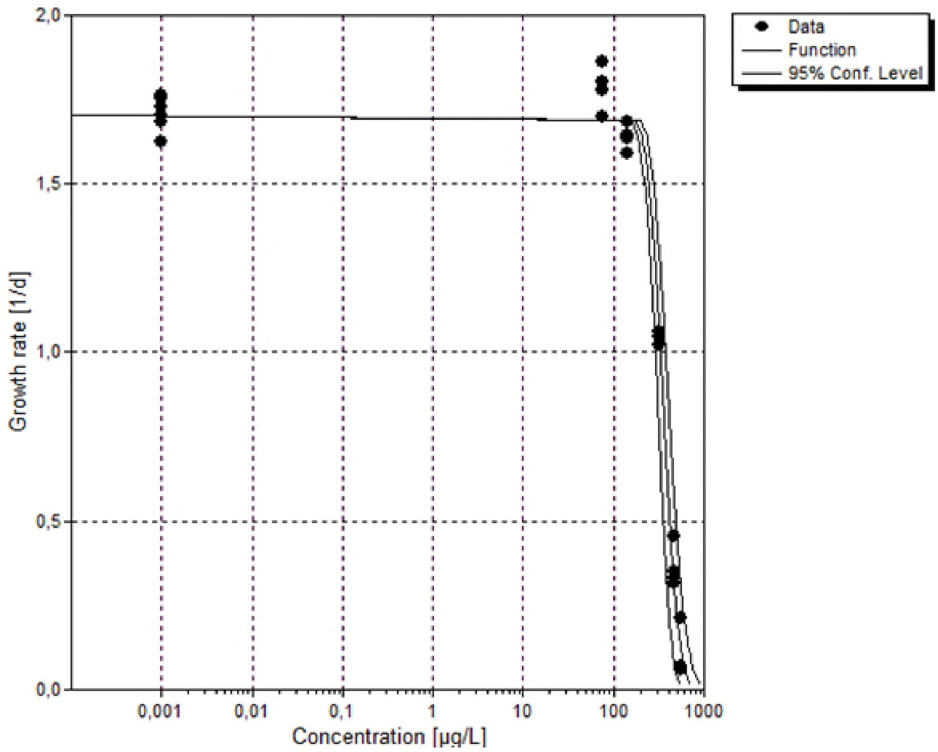
Toxicological endpoint: Growth rate

Figure 36: Growth rate of *S. marinoi* and test item 4-ADNT over exposure time



Source: own illustration, German Environment Agency

Figure 37: Growth rate of *S. marinoi* over test item 4-ADNT concentration



Source: own illustration, German Environment Agency

3.6.1.2 Test 2 of 2

Schelzig, S. (2023e): Prüfung der Toxizität von 4-Amino-2,6-dinitrotoluol auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no.C27Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C27Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124182?sv=

The results of the testing of the toxicity of 4-ADNT on *Skeletonema marinoi* are shown in the tables 30, 31 and 32 and figures 38, 39, 40, 41 and 42 for the second test undertaken.

Analytical determination of test item concentration

Table 30: Analytical determination of test item 4-ADNT

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	500.0	71.668	14.3
2	1000.0	143.188	14.3
3	2000.0	256.172	12.8
4	3000.0	393.177	13.1
5	4000.0	523.375	13.1
6	5000.0	646.302	12.9
7	6000.0	732.502	12.2

Effect concentration

Table 31: Effect concentration for test item 4-ADNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	236.04	296.85
lower CL	222.94	283.18
upper CL	249.91	311.18
EC_{50}	318.71	485.41
lower CL	298.55	459.43
upper CL	340.49	512.76
LOEC	256.17	256.17
NOEC	143.19	143.19

Inhibition of yield and growth rate

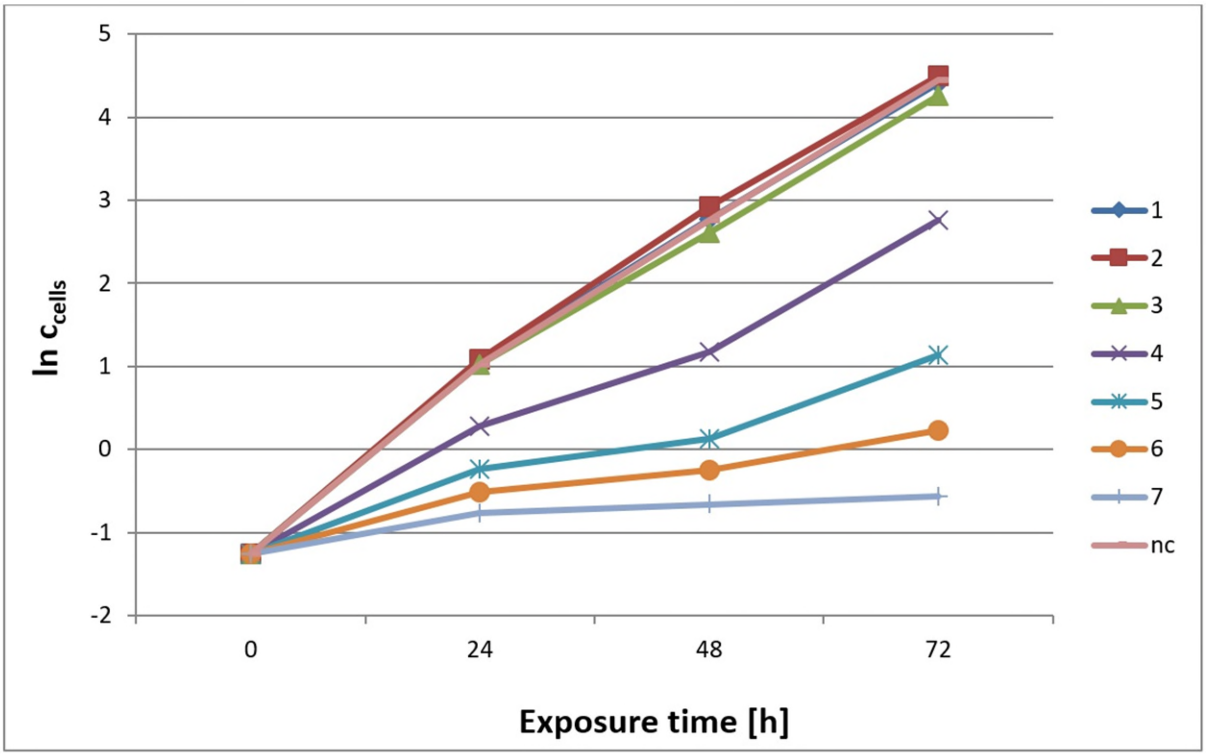
Table 32: Inhibition of yield and growth rate over 72 hours by test item 4-ADNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	72.668	3.5	0.6
2	143.188	-5.0	-0.8
3	256.172	17.0	3.3
4	393.177	81.8	29.7
5	523.375	96.7	58.4
6	646.302	98.9	74.3
7	732.502	99.7	87.8

Dose-response relationships

Cell concentration over exposure time

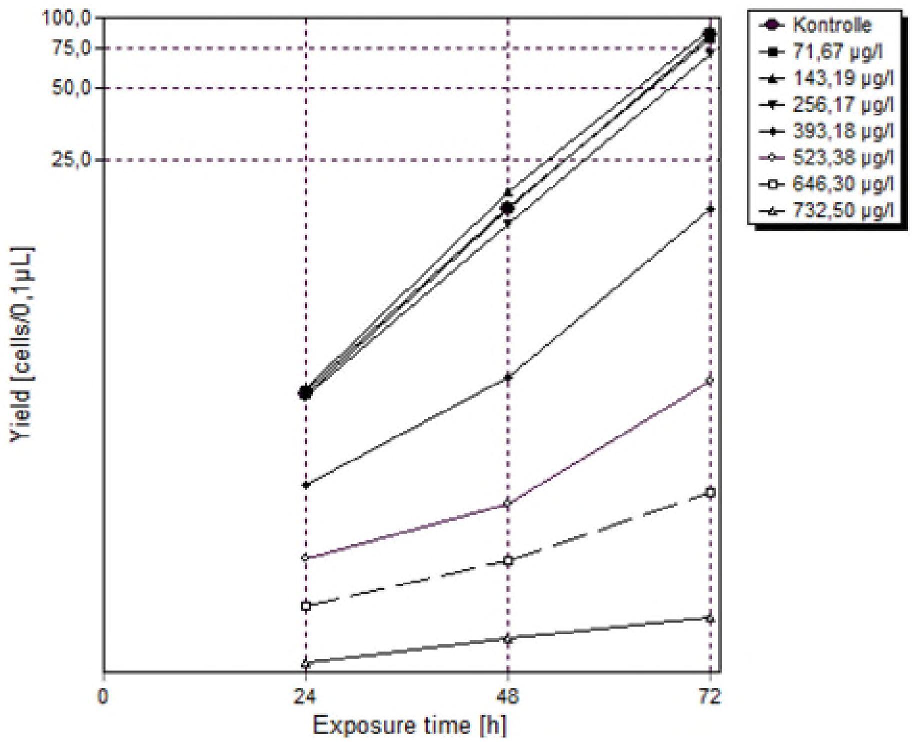
Figure 38: Cell concentration of *S. marinoi* in relation to test item 4-ADNT concentration and exposure time



Source: own illustration, German Environment Agency

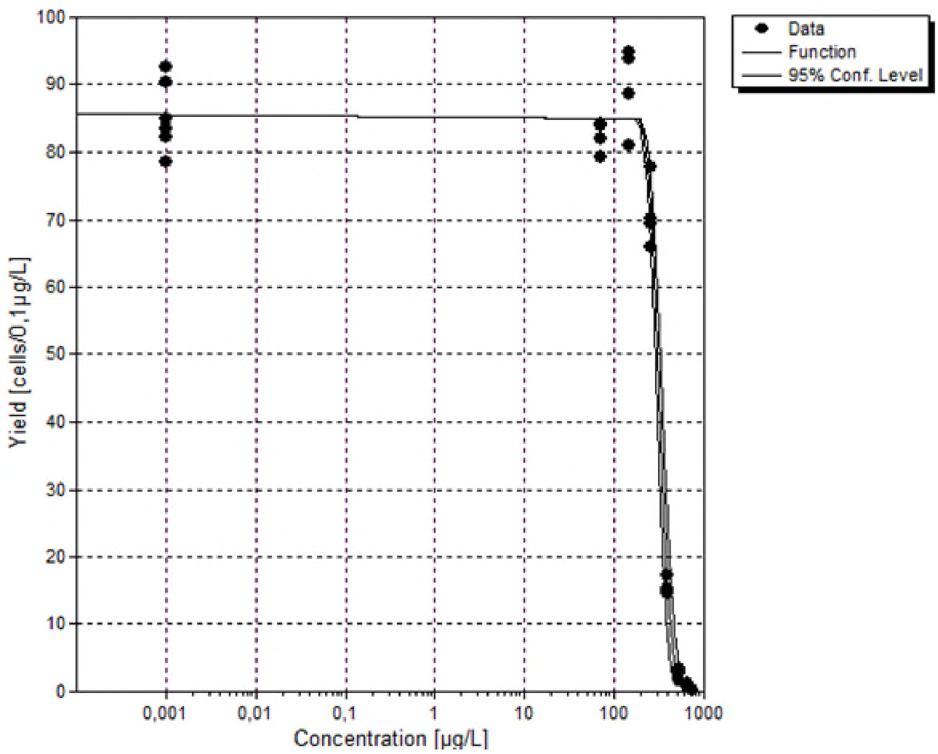
Toxicological endpoint: Yield

Figure 39: Yield of *S. marinoi* and test item 4-ADNT over exposure time



Source: own illustration, German Environment Agency

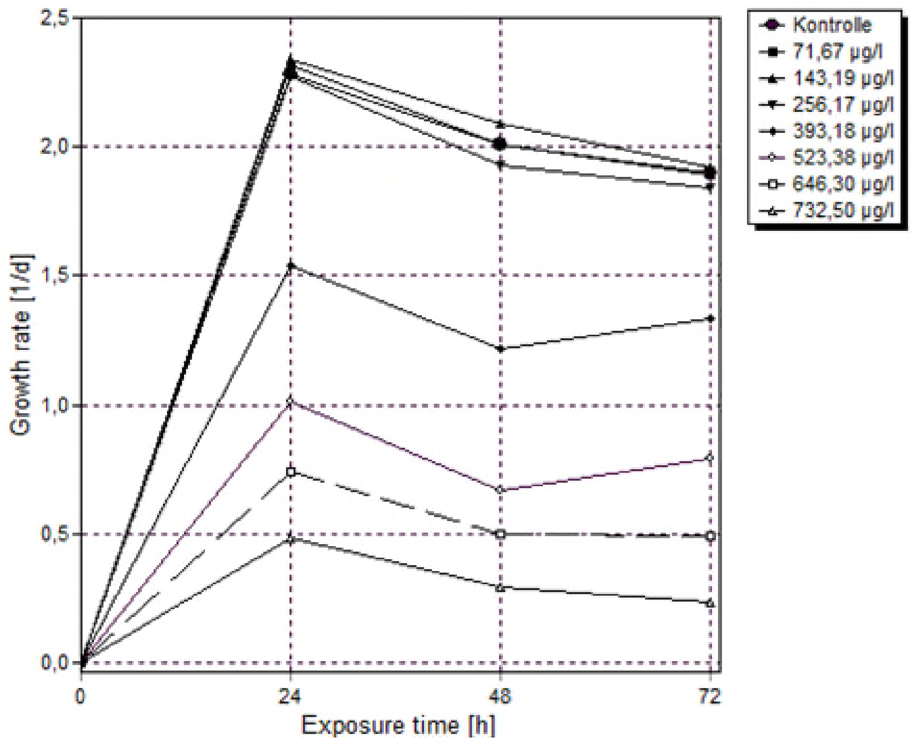
Figure 40: Yield of *S. marinoi* over test item 4-ADNT concentration



Source: own illustration, German Environment Agency

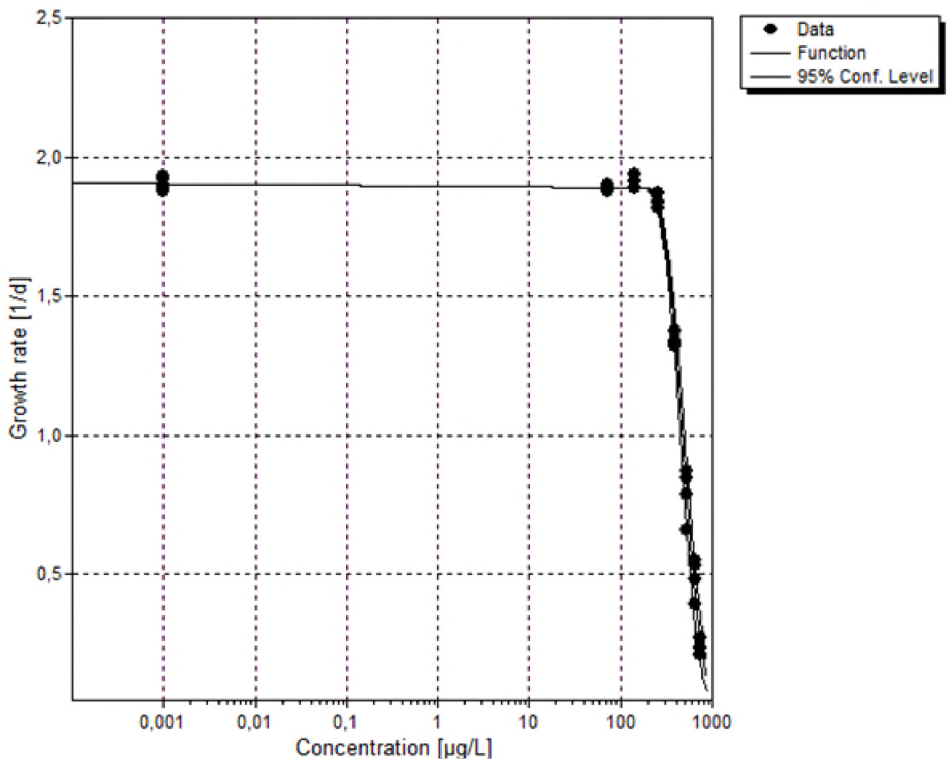
Toxicological endpoint: <u>Growth rate</u>

Figure 41: Growth rate of *S. marinoi* and test item 4-ADNT over exposure time



Source: own illustration, German Environment Agency

Figure 42: Growth rate of *S. marinoi* over test item 4-ADNT concentration



Source: own illustration, German Environment Agency

Geometric mean overall tests with *S. marinoi* and test item 4-ADNT

Due to the two tests with *S. marinoi* and 4-ADNT, the geometric mean was calculated over all tests with *S. marinoi* and test item 4-ADNT for effect concentrations in table 33 and for the inhibition of yield and growth rate in table 34.

Effect concentration

Table 33: Effect concentration (geometric mean) for test item 4-ADNT

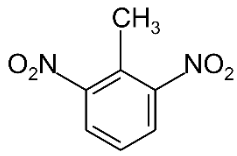
EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	176.3	265.1
lower CL	165.8	244.0
upper CL	187.5	288.0
EC_{50}	257.1	412.9
lower CL	239.9	375.5
upper CL	276.1	454.5
LOEC	> 461.7	191.5
NOEC	≥ 345.2	102.9

Inhibition of yield and growth rate

Table 34: Inhibition (arithmetic mean) of yield and growth rate over 72 hours by test item 4-ADNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	72.84	4.8	-1.9
2	143.15	5.8	1.7
3	287.814	53.1	21.3
4	433.31	90.4	54.3
5	535.912	98.3	76.8
6	667.25	99.5	90.0
7	782.31	100	100.4

3.7 2,6-DNT (2,6-Dinitrotoluene)



3.7.1 *Skeletonema marinoi*

Schelzig, S. (2024): Prüfung der Toxizität von 2,6-Dinitrotoluol auf marine Kieselalgen der Art *Skeletonema marinoi* (Test no. C29Sm). Umweltbundesamt, Ökotoxikologielabor, Prüfungscode C29Sm.

https://recherche.chemikalieninfo.de/etox/zitat/124179?sv=

The results of the testing of the toxicity of 2,6-DNT on *Skeletonema marinoi* are shown in the tables 35, 36 and 37 and figures 43, 44, 45, 46 and 47.

Analytical determination of test item concentration

Table 35: Analytical determination of test item 2,6-DNT

sample	nominal concentration [µg/L]	true concentration [µg/L]	recovery rate [%]
nc	0	0	-
1	500.0	405.871	81.17
2	2000.0	1717.715	85.89
3	3500.0	3207.328	91.64
4	5000.0	4599.903	92.00
5	6500.0	6201.516	95.41
6	8000.0	7520.031	94.00
7	9500.0	9184.268	96.68

Effect concentration

Table 36: Effect concentrations for test item 2,6-DNT

EC_x [µg/L]	inhibition of yield	inhibition of specific growth rate
EC_{10}	1787.749	5259.918
lower CL	1426.454	4949.303
upper CL	2240.553	5590.027
EC_{50}	3970.259	7844.424
lower CL	3076.377	7258.090
upper CL	5150.176	8454.498
LOEC	≤ 405.871	≤ 405.871
NOEC	< 405.871	< 405.871

Inhibition of yield and growth rate

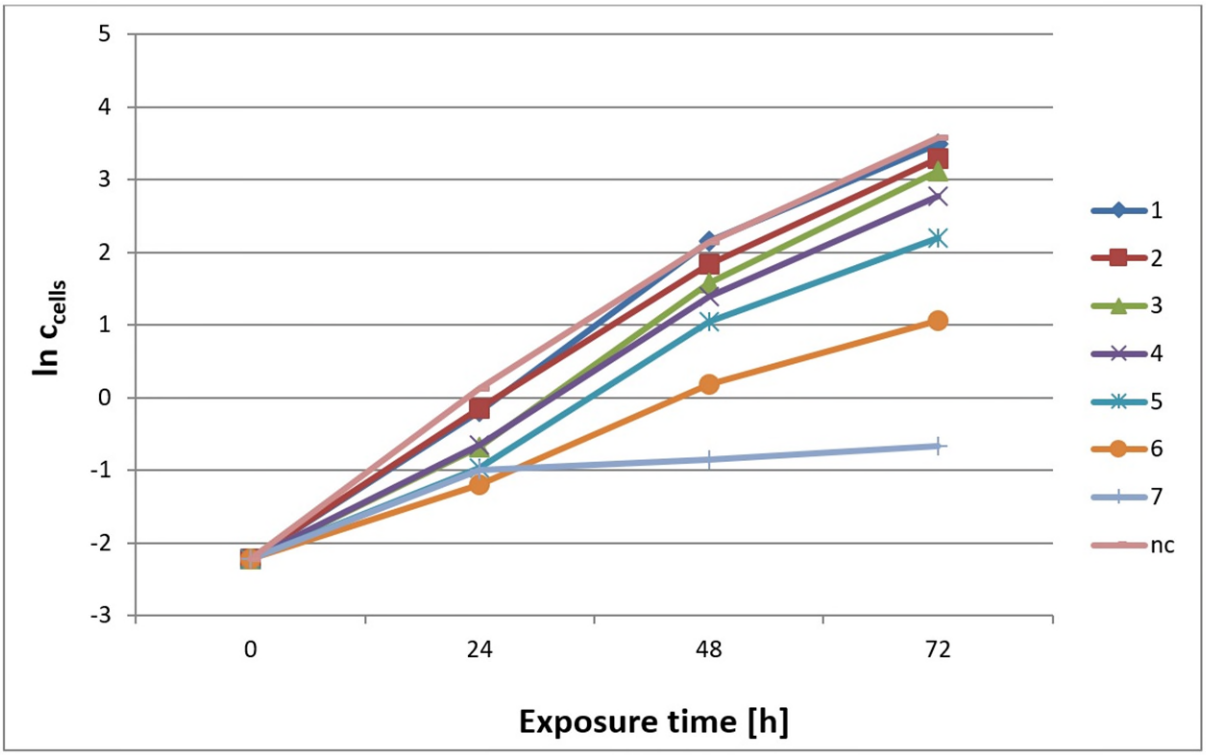
Table 37: Inhibition of yield and growth rate over 72 hours by test item 2,6-DNT

sample	test item concentration [µg/L]	inhibition [%] yield	inhibition [%] growth rate
nc	0	-	-
1	405.871	9.1	1.6
2	1717.715	15.9	5.2
3	3207.328	37.1	8.0
4	4599.903	55.7	14.0
5	6201.516	75.2	23.9
6	7520.031	92.3	43.7
7	9184.268	98.9	73.5

Dose-response relationships

Cell concentration over exposure time

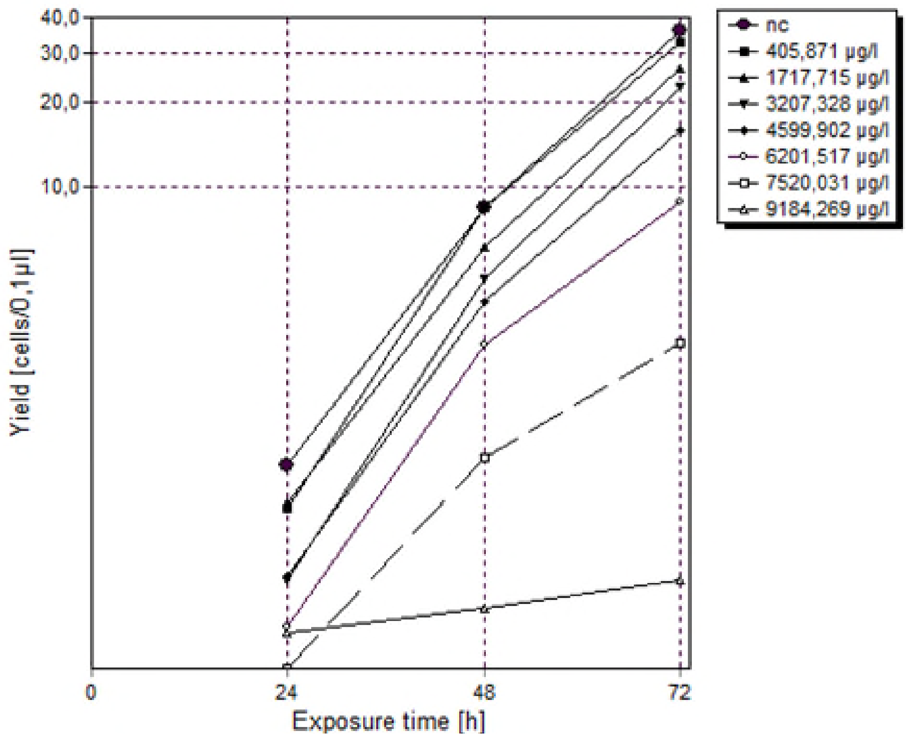
Figure 43: Cell concentration of *S. marinoi* in relation to test item 2,6-DNT concentration and exposure time



Source: own illustration, German Environment Agency

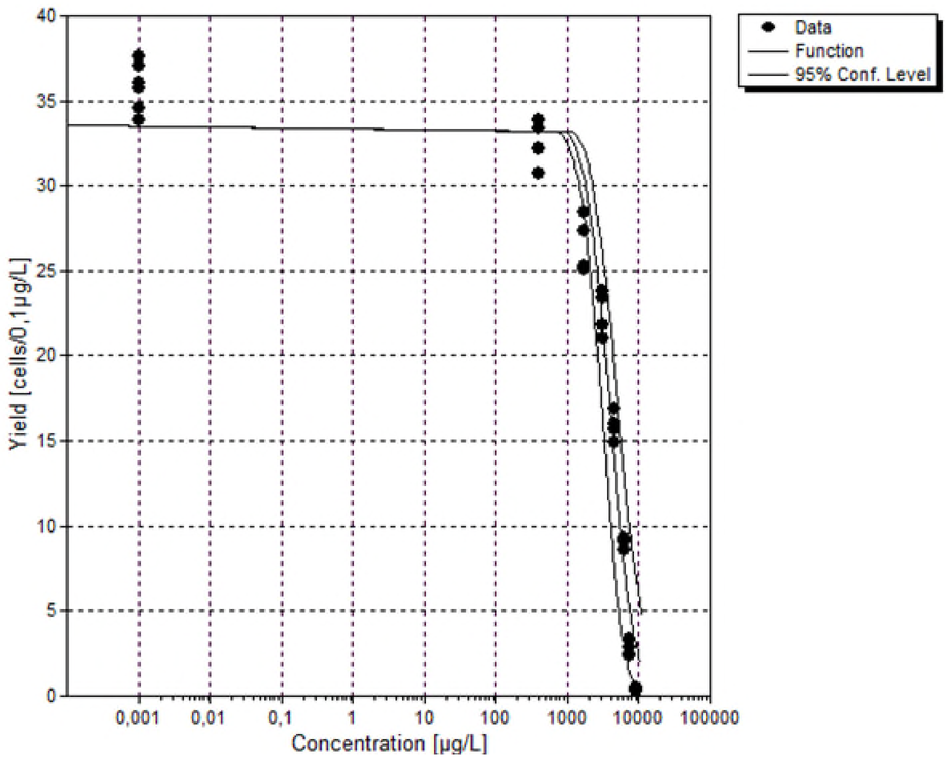
Toxicological endpoint: <u>Yield</u>

Figure 44: Yield of *S. marinoi* and test item 2,6-DNT over exposure time



Source: own illustration, German Environment Agency

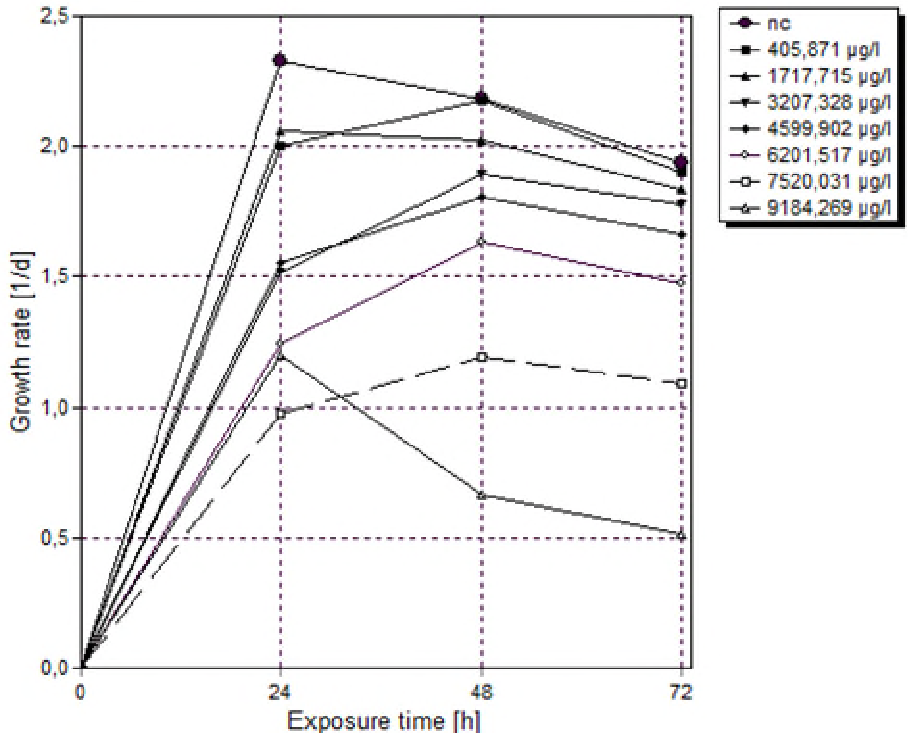
Figure 45: Yield of *S. marinoi* over test item 2,6-DNT concentration



Source: own illustration, German Environment Agency

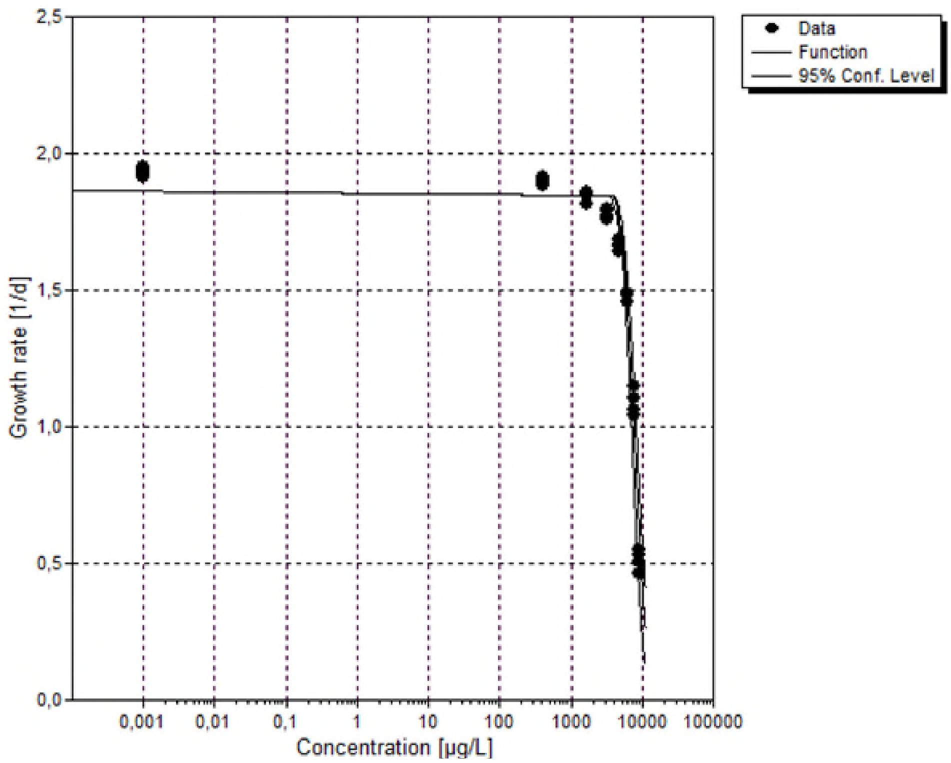
Toxicological endpoint: <u>Growth rate</u>

Figure 46: Growth rate of *S. marinoi* and test item 2,6-DNT over exposure time



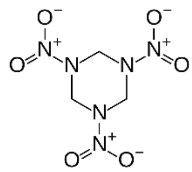
Source: own illustration, German Environment Agency

Figure 47: Growth rate of *S. marinoi* over test item 2,6-DNT concentration



Source: own illustration, German Environment Agency

3.8 RDX (Hexogen)

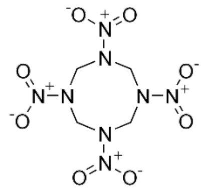


RDX (abbreviation of "*Research Department eXplosive*" or "*Royal Demolition eXplosive*") was deselected from testing because studies with freshwater algae such as *Selenastrum capricornutum* have shown that even at its solubility limit of 36.7 mg/L in algal assay media, there was no EC_{50} determinable, with a maximum observed reduction in cell density of only 38 % after 96 hours (Burton et al., 1994). No effect concentrations were reported for *Microcystis aeruginosa, Anabaena flos-aquae*, and the freshwater diatom *Navicula pelliculosa* (Bentley et al., 1977).

For *Navicula pelliculosa*, the highest observed reduction in cell density was 17 % at 32 mg/L (nominal) and after recalculation of the data by Sullivan et al. (1979) the LOEC determined to be 10 mg/L (nominal).

Given these findings, marine diatoms would likely exhibit similar sensitivity, making it highly improbable that an EC_{50} below the solubility limit could be observed under standard testing conditions.

3.9 HMX (Octogen)



Similarly to RDX, HMX (abbreviation of "*High Melting Explosive*", "*High-velocity Military Explosive*" or "*High-Molecular-weight RDX*") also showed very low toxicity to freshwater algae.

Testing on the same species as in their study on RDX, Bentley et. al. (1977) found no adverse effects at concentrations up to 32 mg/L, with no determinable EC_{50} values.

4 Discussion of toxicity tests marine diatoms

4.1 Derivation of a correlation between cell concertation and fluorescence intensity

As described in chapter 2 “Materials and Methods for toxicity tests with marine diatoms”, to perform time efficient toxicity tests with algae it is warranted to use so called surrogate parameters such as absorbance (optical density) or fluorescence intensities to determine the algae concentration instead of counting algae.

Therefore, a correlation between cell concentration and fluorescence intensity was determined for *P. tricornutum* and *S. marinoi*.

To optimize the fit of the regression, particularly in the lower ranges of the fluorescence intensity and to minimize the occurrence of (theoretically possible) negative algae concentrations, the linear regression was fitted through the origin (intercept = 0).

P. tricornutum	$y = 0.0835 \left[\frac{mL}{cells}\right] * x \left[\frac{cells}{mL}\right] + 0$
S. marinoi	$y = 0.1274 \left[\frac{mL}{cells}\right] * x \left[\frac{cells}{mL}\right] + 0$

All validity criteria according to DIN EN ISO 10253 (2016) were met with both correlations.

Furthermore, the regression slops were within the range of other correlations for *P. tricornutum* and *S. marinoi* derived in prior studies at the testing facility of the German Environment Agency.

4.2 Reference testing

In order to ensure the integrity of the test systems and to verify that the chosen test organisms respond with the expected sensitivity to test substances, reference tests with the reference substance 3,4-dichlorophenol (3,4-DCP) were conducted prior to the ecotoxicological experiments, in accordance with DIN EN ISO 10253 (2016).

The results of these reference tests were compared with the outcomes of an international laboratory proficiency test conducted as part of the validation of DIN EN ISO 10253 (results reported by Baudo (2015)), as well as with the results of reference tests previously conducted at the testing facility.

The international laboratory proficiency test used *Skeletonema costatum*. Based on molecular biological comparative analyses and a subsequent taxonomic revision, the diatom previously known as *S. costatum* has been reclassified as *S. marinoi* as of March 2, 2022.

The results of the reference tests unequivocally confirm the integrity of the test system and the expected sensitivity of the diatom species used as seen by the following Table 38 comparing the results of the reference tests with those of the international laboratory proficiency test conducted within the framework of the DIN EN ISO 10253 validation.

Table 38: EC(r)$_{50}$ for test item 3,5-DCP

Species	Inhibition of growth rate by 3,4-DCP EC(r)$_{50}$ [mg/L]	
	reference tests at the German Environment Agency	International laboratorz profiency tests reported by Baudo (2015)
S. marinoi	1.3	1.6
P. tricornutum	2.3	2.7

4.3 Difficulties in cultivating and testing *Phaeodactylum tricornutum*

During the course of the experimental work, difficulties in the cultivation and testing of *P. tricornutum* occurred repeatedly and with increasing frequency.

For unknown reasons, the growth rates in the algal cultures of *P. tricornutum* varied highly between different experiments, which repeatedly led to invalid test results. Even the repeated acquisition of the algal culture from SAG Göttingen could not resolve that issue sustainably. Likewise, attempts using modified growth media and seawater approaches (both natural and artificial) were unsuccessful.

Therefore, towards the end of the second project year, it was decided to conduct the experimental trials only with *S. marinoi*.

This species was suitable to generate valid test results throughout the entire project period. The growth rates of *S. marinoi* were consistently sufficient without high variations. Additionally, the comparison of both species with TNT showed that *S. marinoi* is more sensitive than *P. tricornutum*. It can be assumed that this finding can very likely be extended to the TNT metabolites 2-ADNT and 4-ADNT.

4.4 Ecotoxicity of tested diatom species to munition compounds

In the present study, munition compounds and their metabolites were investigated, and their ecotoxicological potential was assessed with respect to two marine diatom species: *P. tricornutum* and *S. marinoi*.

The results demonstrate that all of the investigated munition compounds and metabolites exhibited toxicity towards the diatoms used (Table 39 and Fig. 48).

S. marinoi showed a significantly higher sensitivity to TNT compared to *P. tricornutum*.

Overall, TNT, followed by its metabolites, 2-ADNT and 4-ADNT, exhibited the highest ecotoxicological potential of all tested munition compounds in this study.

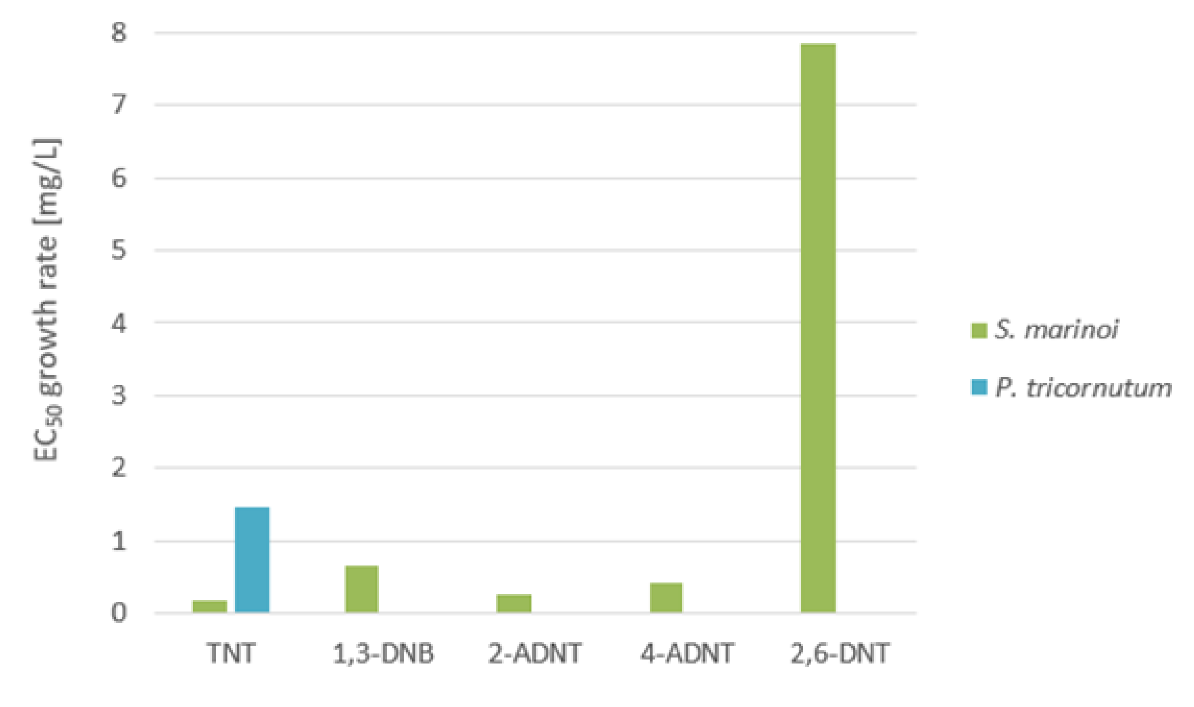
1,3-DNB showed a toxicity similar to that of the TNT metabolites 2-ADNT and 4-ADNT and the toxicity of 2,6-DNT was approximately a full order of magnitude lower.

According to literature data (see Table 39), RDX and HDX only become toxic to aquatic primary producers at concentrations in the high mg/L range.

Table 39: Summarized results of the ecotoxicological testing with *S. marinoi* and *P. tricornutum*

tested substance	*S. marinoi*				*P. tricornutum*			
	Yield		growth rate		yield		growth rate	
	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}
	mg/L				mg/L			
TNT	0.06	0.11	0.13	0.18	0.04	0.41	1.32	1.47
2-ADNT	0.21	0.23	0.23	0.26	/	/	/	/
4-ADNT	0.18	0.26	0.27	0.41	/	/	/	/
1,3-DNB	0.46	0.53	0.63	0.65	/	/	/	/
2,6-DNT	1.79	3.97	5.26	7.84	/	/	/	/
RDX	according to literature data, EC_{50} above solubility limit (> 37 mg/L) (Burton et al., 1994; Bentley et al., 1977; Sullivan et al., 1979)							
HMX	according to literature data, no adverse effects at concentrations up to 32 mg/L, with no determinable EC_{50} values (Bentley et al., 1977)							

Figure 48: Comparison auf EC_{50} growth rates ($EC(r)_{50}$) for 5 munition compounds and related metabolites with data for the marine diatoms *S. marinoi* and *P. tricornutum*



Source: own illustration, German Environment Agency

The applied testing procedure according to DIN EN ISO 10253 (2016) allows statements regarding the sensitivity of the test organisms used and the extent of the toxicological potential of the investigated chemical substances.

Statements about modes of action are not possible with these test methods because using growth and survival as endpoints for toxicity.

Further studies including biochemical and molecular biological parameters might provide more insights into the respective modes of action triggered by munition compounds and their metabolites.

4.5 Comparison of toxicity with other species – SSDs

4.5.1 SSD as statistical method

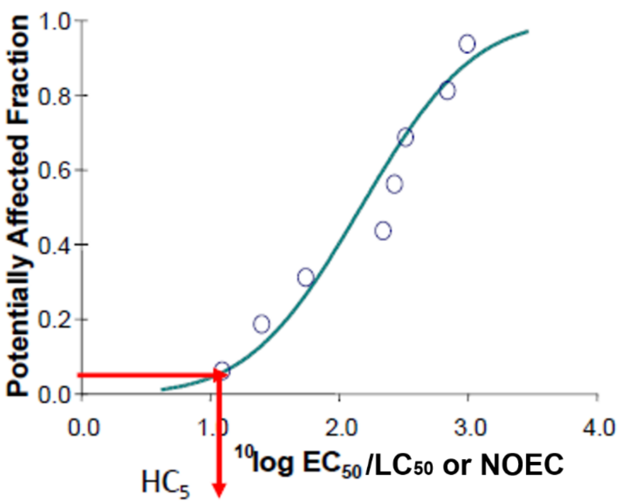
Species Sensitivity Distributions are a statistical method used to assess the sensitivity of different species to a particular environmental stressor or pollutant.

SSDs represent a distribution of the sensitivities of various species and provide a basis for estimating the potential risks posed by a contaminant or chemical substance to different species within an ecosystem.

SSDs are typically created by examining the toxicity of a specific pollutant across a broad range of species. Toxicity data from a variety of species are collected and represented in a distribution, with the **sensitivity of species** plotted on the x-axis and the **frequency of sensitivities** on the y-axis. This could, for example, represent the response of fish, invertebrates, algae, and other aquatic organisms to a particular contaminant.

Figure 49 is illustrating the variation in sensitivity among different species to a particular chemical substance. HC_5 stands for the Hazardous Concentration for 5% of species. It is the concentration of a chemical substance at which 5% of the species in the dataset are expected to be affected, meaning it corresponds to the 5th percentile of the SSD curve.

Figure 49: Schematic representation of a Species Sensitivity Distribution (SSD)



Source: ChemSafetyPRO

The SSD can be converted into a cumulative form to estimate the probability that a specific concentration of a pollutant will cause harmful effects to a given species group.

Species Sensitivity Distributions are a valuable tool in environmental science, helping to assess risks to biodiversity and ecosystems. They allow for the systematic comparison and understanding of species sensitivities and the concentrations of pollutants at which they may become problematic for different species in an ecosystem. This method is a key component of ecological risk assessment and the establishment of environmental standards.

4.5.2 Comparison of diatom toxicity of the present study to published SSDs

The results of the present study are compared with the SSDs from Lotufo et al. (2017).

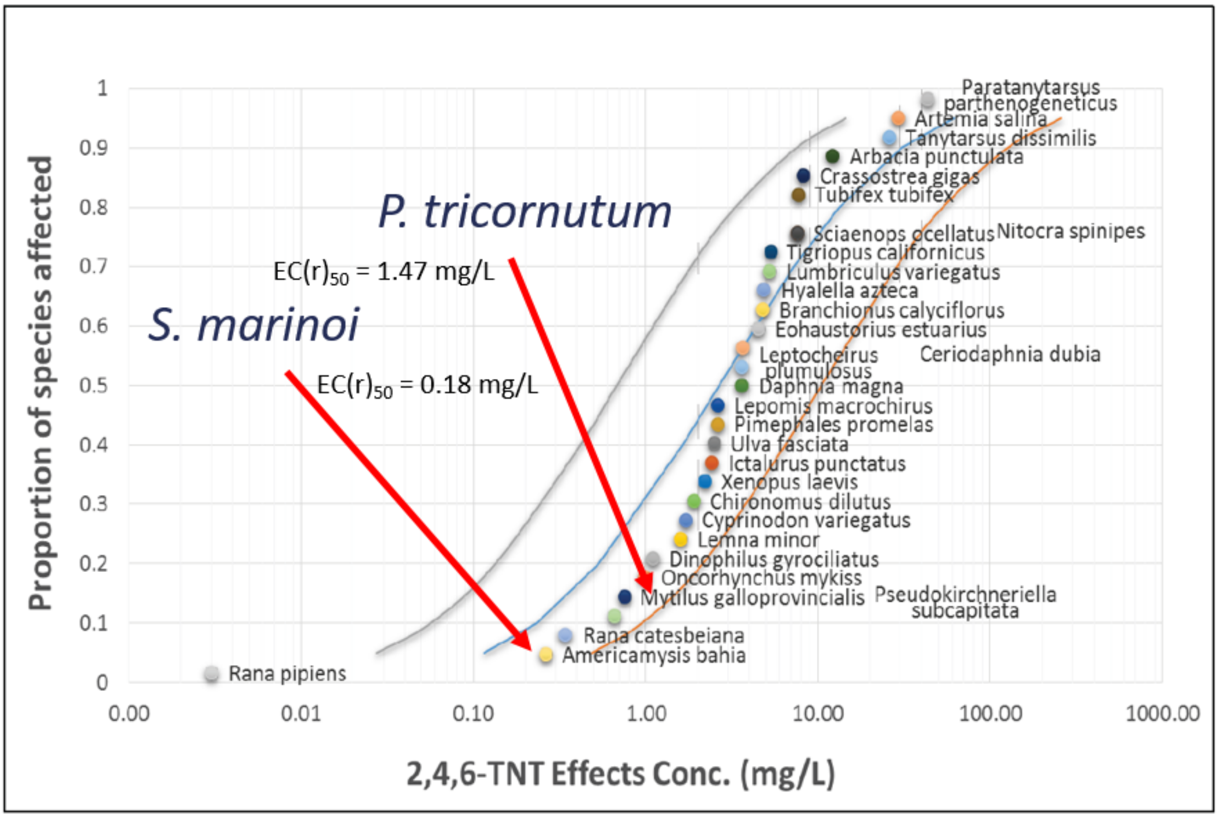
4.5.2.1 SSD for TNT

For TNT, the SSD clearly shows the high sensitivity of the tested diatoms to the munitions compound.

Only the larvae of an amphibian species (*Rana pipiens*) were tested to be more sensitive than the diatom *S. marinoi*.

P. tricornutum also exhibited a relatively high sensitivity to TNT, falling within the approximately 15% of the most sensitive organisms tested in response to TNT.

Figure 50: Species sensitivity distribution for TNT by Lotufo et al. (2017), supplemented with data for *S. marinoi* and *P. tricornutum*



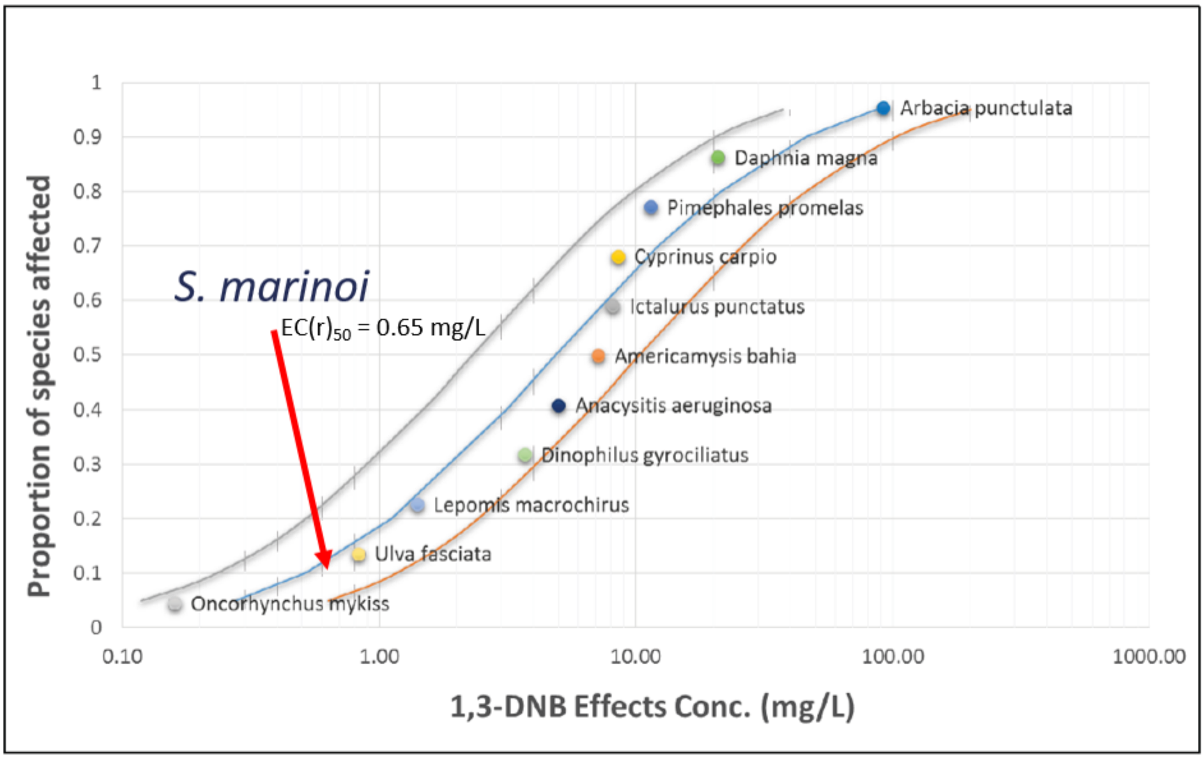
Source: Lotufo et al. (2017) modified by German Environment Agency

4.5.2.2 SSD for 1,3-DNB

S. marinoi also exhibits high sensitivity to the munition compound DNB.

The SSD from Lotufo et al. (2017) only lists rainbow trout (*Oncorhynchus mykiss*) as a species with higher sensitivity to DNB than *S. marinoi*. Thus, *S. marinoi* is also within the most sensitive 15% of all species tested for DNB so far.

Figure 51: Species sensitivity distribution for 1,3-DNB by Lotufo et al. (2017), supplemented with data for *S. marinoi*



Source: Lotufo et al. (2017) modified by German Environment Agency

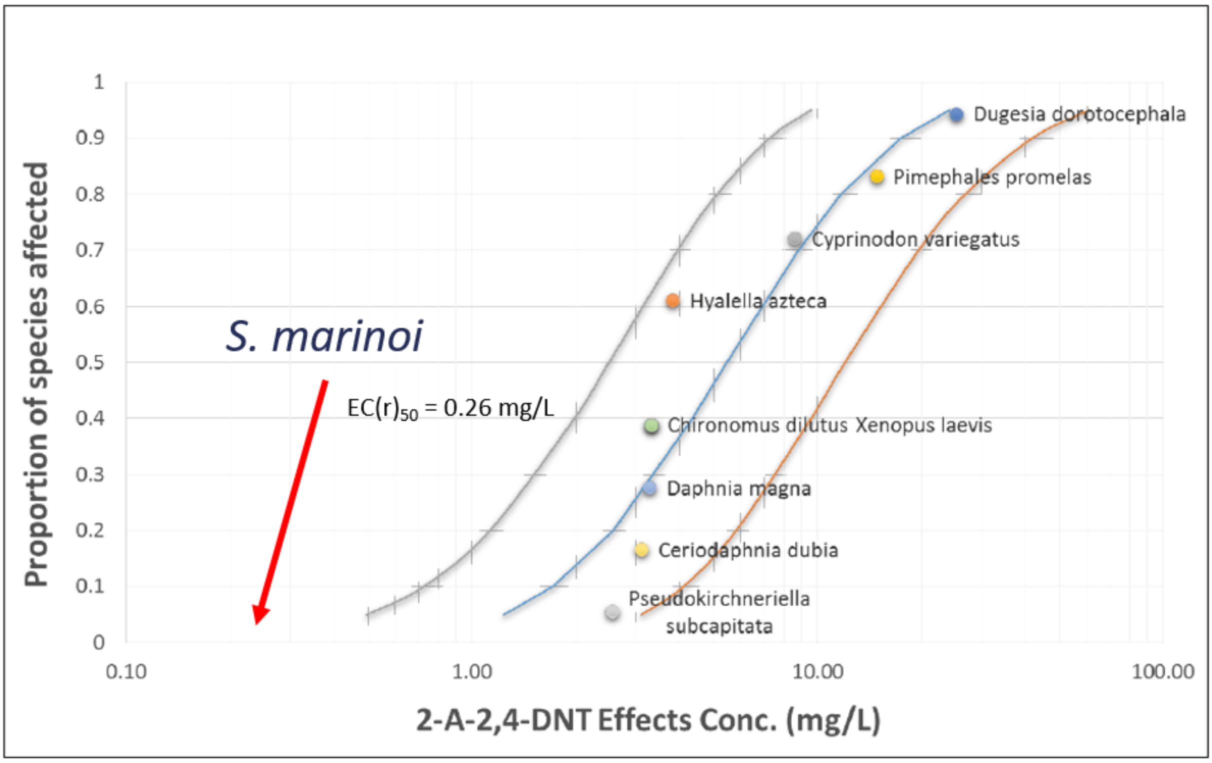
4.5.2.3 SSD for 2-ADNT and 4-ADNT

S. marinoi is by far the most sensitive species investigated to date with respect to the TNT metabolites 2-ADNT and 4-ADNT.

According to SSDs published by Lotufo et al. (2017), *S. marinoi* exhibits sensitivities approximately one order of magnitude higher than those of the species previously listed as the most sensitive ones concerning 2-ADNT (the green algae *Pseudokirchneriella subcapitata* (Fig. 52)) and 4-ADNT (the planarian *Dugesia dorotocephala*[8] (Fig. 53)).

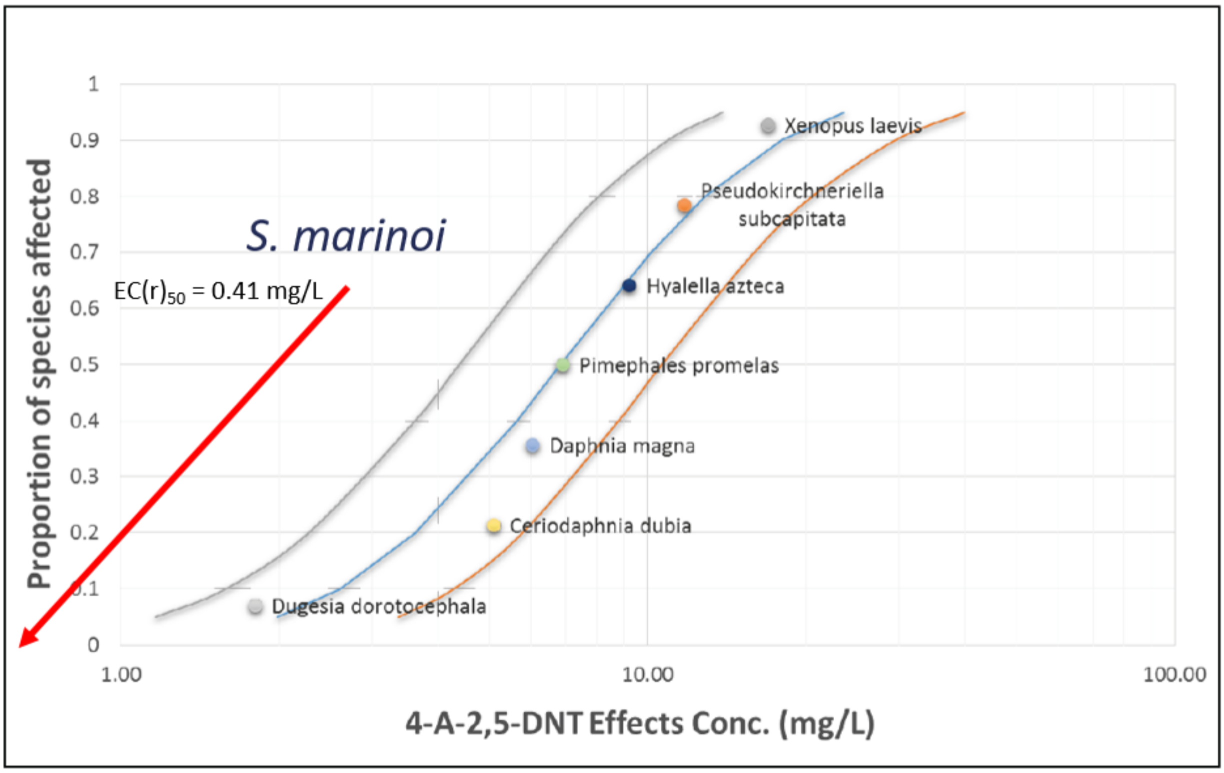
[8] Currently known as *Girardia dorotocephala*

Figure 52: Species sensitivity distribution for 2-ADNT by Lotufo et al. (2017), supplemented with data for *S. marinoi*



Source: Lotufo et al. (2017) modified by German Environment Agency

Figure 53: Species sensitivity distribution for 4-ADNT by Lotufo et al. (2017), supplemented with data for *S. marinoi*



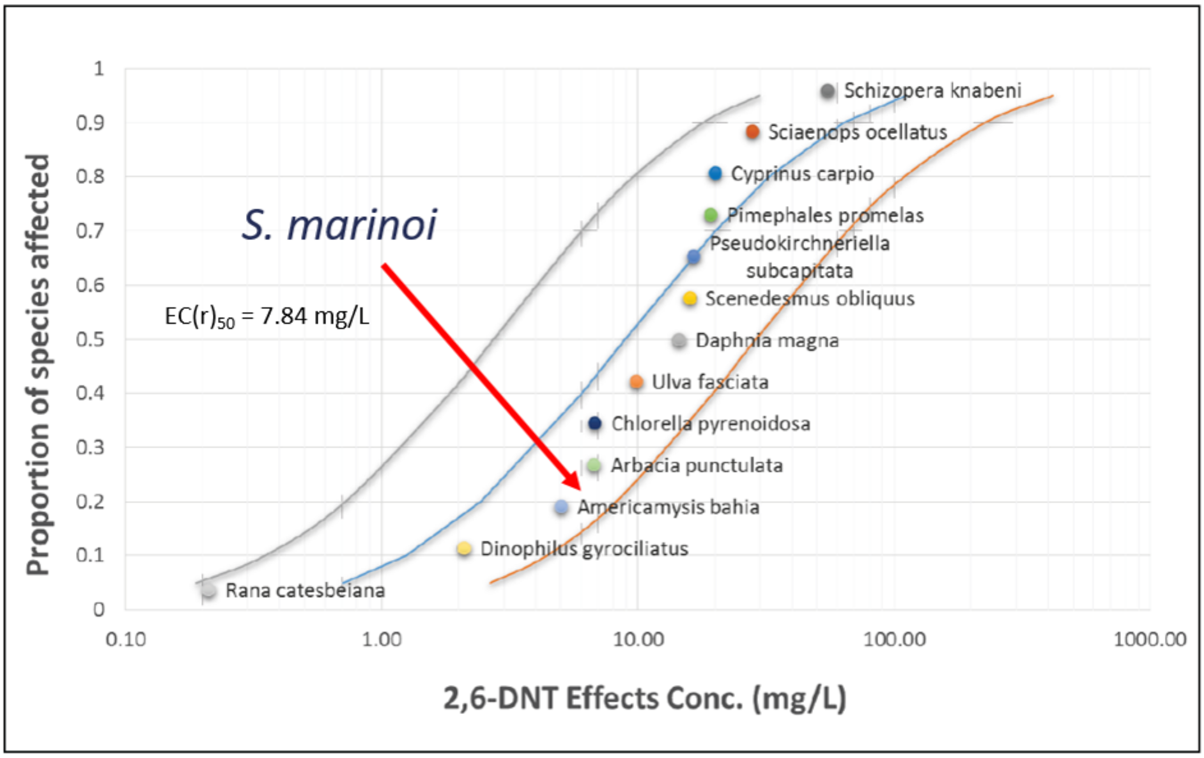
Source: Lotufo et al. (2017) modified by German Environment Agency

4.5.2.4 SSD for 2,6-DNT

As already described in Chapter 4.4, the munition compound 2,6-DNT exhibited the lowest, yet still a clearly pronounced, toxic potential among all the explosives and their metabolites investigated in this study.

Compared to the results compiled by Lotufo et al. (2017) in their SSD for 2,6-DNT, the diatom *S. marinoi* is among the most sensitive 25 % of all species previously tested.

Figure 54: Species sensitivity distribution for 2,6-DNT by Lotufo et al (2017), supplemented with data for *S. marinoi*



Source: Lotufo et al. (2017) modified by German Environment Agency

4.5.2.5 Conclusion on sensitivity of tested diatoms

The detailed comparison of the diatom species investigated in the present study with the results published by Lotufo et al. (2017) through numerous SSDs clearly shows that diatoms are very sensitive to munition compounds and their metabolites.

This finding is particularly significant in the context that diatoms, as primary producers, are at the base of the marine food web. The toxicity endpoints found in our study can be used to derive Environmental Quality Standards, which are used as limit values for measured environmental concentrations.

Negative growth impacts on diatom populations from leaking munition compounds and their metabolites might have far-reaching consequences for populations of organisms at higher trophic levels due to reduced availability of algal food.

5 Environmental Quality Standards

5.1 Legal framework for Environmental Quality Standards

Environmental Quality Standards (EQS), as defined by the Water Framework Directive (WFD) (European Commission, 2000), refer to the legally established concentration limits for specific pollutants or substances in surface waters that aim to protect the aquatic environment's ecological and chemical status.

These standards are designed to safeguard biodiversity, water resources, and human health by ensuring that concentrations of potentially harmful substances and do not exceed thresholds that would cause detrimental effects on ecosystems or impair water uses, such as drinking water supply, recreation, aquaculturally and fishery activities. The EQS are applied to various parameters, including chemical pollutants (e.g., heavy metals, pesticides or even explosives) and biological indicators, in order to maintain or achieve "Good Status" of surface waters by the designated timeline of 2027.

5.2 Tentative Quality Standard for TNT for protecting pelagic species

Based on the toxicity data of this study and further published data from other toxicity studies, draft EQS values for TNT have been derived and the detailed TNT EQS data sheets has been published (Schudoma et al., 2025). The procedure and results are summarised and discussed here.

According to Technical Guidance Document (TGD) No. 27 (European Commission, 2018), two EQSs are required for the water compartment to cover both long-term and short-term exposures to a chemical. Whilst derivation of the EQS typically employs chronic toxicity data, the MAC-EQS always relies on acute data.

To derive a **$MAC_{freshwater, eco}$** for TNT, acute effect data are available for bacteria, algae, invertebrates, fish and other species (see Figure 50). The lowest acute test result exists for the algae *S. marinoi* (growth rate 72 h = ErC_{50}: 182 µg/L). An assessment factor (AF) = 100 is used to derive the EQS for the maximum concentration.

$MAC_{freshwater, eco}$ = 182 µg/L * 0.01 = 1.8 µg/L

To derive an **$AA\text{-}QS_{freshwater, eco}$** for TNT, data of tests with long-term exposure are available for bacteria, algae, invertebrates, fish and amphibia. The lowest test result for fish was the LOEC of 40 µg/L for the survival (60 d) in the F1 generation of the fish *Pimephales promelas* (Bailey et al. 1985). The LOEC is divided by 2 to calculate a NOEC of 20 µg/L for fish *P. promelas*. The lowest test result for amphibia was determined for tadpoles of frog *Rana pipiens* (28 d/survival/LOEC = 3 µg/L) by (Stanley 2015). For *R. pipiens* a NOEC is extrapolated LOEC/2 = 1.5 µg/L. As NOEC values with long-term test are existing, an AF = 10 is used to calculate an **$AA\text{-}QS_{freshwater, eco}$**.

$AA\text{-}QS_{freshwater, eco}$ = 1.5 µg/l * 0.1 = 0.15 µg/L

Since there are no differences in the sensitivity of freshwater and marine organisms, and the lowest test result comes from a freshwater species, the values can also be adopted for the marine area (Table 40).

Table 40: Tentative Quality Standards for TNT in water

Tentative QS_{water}	Relevant study for derivation of EQS	Assessment factor	Tentative QS
$MAC_{freshwater, eco}$	*Skeletonema marinoi*/72 h ErC_{50} 185 µg/L	100	1.8 µg/L
$MAC_{saltwater, eco}$		100	1.8 µg/L
$AA\text{-}QS_{freshwater, eco}$	*Rana pipiens* (28 d/survival/NOEC = 1.5 µg/L)	10	0.15 µg/L
$AA\text{-}QS_{saltwater, eco}$		10	0.15 µg/L

5.3 Tentative QS for TNT in sediment

TNT exhibits weak sorption to sediments. Kd values on the order of 10 L/kg were reported by (Beck et al. 2018). Using a standard organic carbon content of 10% for sediments a Kd value of 10 L/kg is comparable to Koc of 100 L/kg (Koc = (Kd * 100) / % organic carbon). A trigger value of Koc ≥1000 or Kow ≥1000 is given in TGD EQS to perform a sediment effects assessment. For TNT a Koc = 44.29 at 20°C is reported in the ECHA brief profile for TNT, which below the trigger value.

The Equilibrium Partitioning (EqP) method is therefore not used to estimate the QSsediment (European Commission, 2018). Nevertheless, TNT and its metabolites were found in sediments and some results from sediment ecotoxicity tests exist.

For TNT and its transformation products, rapid transformation, disappearance of transformation products and distribution into overlying water after initiation of whole sediment toxicity testing were reported (Lotufo et al. 2021). The rapidly fluctuating concentrations and the transformation products in sediment pose unique challenges for the development of accurate toxicity data to assess risks to biota at sites with contaminated sediments. The toxicity of TNT-enriched sediment to marine polychaete, estuarine amphipod, freshwater midge, amphipod, oligochaete and estuarine fish occurred over a wide concentration range from 37 to 508 mg/kg (Lotufo et al. 2021). Due to the very heterogeneous data situation, it is currently not possible to derive a reliable quality standard for the protection of the benthic community.

5.4 Deriving a biota standard to protect wildlife from secondary poisoning QS_{biota} (EQS for TNT in biota)

The bioaccumulation of TNT is low (*Pimephales promelas*, 4.97 L/kg ww, (Yoo et al. 2006)). The reliable BCF values for fish or other aquatic organisms are below the trigger value of 100 L/kg in TGD EQS to derive a QS_{biota}, secondary poisoning (Table 41). Therefore, no value for QS_{biota} was derived.

Table 41: Toxicity data for secondary poisoning of top predators with TNT

Species group	Effect value	Master reference
Mammalian oral toxicity	Mouse/oral/duration/Endpoint LD_{50} at 660 mg/kg	(Haas and Thieme 1996)
	Rattus rattus/oral/duration/Endpoint LD_{50} at765 mg/kg	(Haas and Thieme 1996)
Avian oral toxicity	Colinus virginianus/oral/90 d LOAEL: 178 mg/kg bw/day NOAEL: ≥ 7mg/kg bw/day	(Gogal Jr., Johnson et al. 2002)

5.5 Protection of humans against adverse health effects from consuming contaminated fisheries products

Human exposure to TNT occurs primarily through occupational settings, such as in munitions manufacturing or military activities, but environmental contamination can also lead to exposure through drinking water, soil, or food. The carcinogenic potential of TNT and its metabolites has raised concerns, as several studies have suggested a possible link between TNT exposure and increased risks of cancer, particularly in workers involved in the production of explosives. TNT considered a hazardous substance due to its toxic properties. Substance may cause cancer and is suspected of damaging fertility of the unborn child and is suspected of causing genetic defects (https://echa.europa.eu/de/brief-profile/-/briefprofile/100.003.900). Although the bioaccumulation of TNT in fish is low (*Pimephales promelas* 4.97 L/kg ww), a quality standard has been derived to protect human health (Table 42).

Table 42: Data used to calculate a tentative Quality Standard to protect human health via consumption of fishery products

Data	Effect value	Master reference
Mammalian oral toxicity	26-week Dog Feeding Study; LOAEL: 0.5 $mg.kg^{-1}{}_{bw}.d^{-1}$	(IRIS)
CMR	Oral Slope Factor: 3.0 10^{-2} per $mg.kg^{-1}{}_{bw}.d^{-1}$	(IRIS)

QS_biota, hh, food = 0.2 x TL x 70 / 0.115 = 0.2 x TL / 0.00163

Allocation factor = 0.2

TL = threshold level, human health (ADI, MTR, TDI [µg/kg bw/d])

Body weight = 70 [kg]

Fish consumption = 0.115 [kg/d]

Fish consumption / Body weight = 0.00163 [kg/kg/d]

Table 43: Calculation for toxic effect of TNT

Tentative $QS_{biota, hh}$	Relevant study for derivation of $QS_{biota, hh}$	Assessment Factor	Tentative $QS_{biota, hh}$
Human health	TL = 0.5 µg/kg bw/d (RfD, IRIS)	1000	60 $\mu g.kg^{-1}{}_{biota\ ww}$

Table 44: Calculation for carcinogenic effects of TNT

Tentative $QS_{biota, hh}$	Relevant study for derivation of $QS_{biota, hh}$	Risk Level	Tentative $QS_{biota, hh}$
Human Health	Oral Slope Factor: 3.0 10^{-2} per $mg.kg^{-1}$ bw. d^{-1} TL = 0.0333 µg/kg bw /d	10^{-6}	4.1 $\mu g.kg^{-1}{}_{biota\ ww}$

The bioconcentration factor (BCF) value for *Pimephales promelas* 4.97 L/kg ww is used to calculate a corresponding water concentration for **$QS_{water\ biota,\ hh}$** of 0.8 µg/L. This value should only be used to compare the **Tentative $QS_{biota,\ hh}$** with other **Tentative QS_{water}**. (Yoo et al. 2006)

Table 45: Effects of human health via consumption of drinking water

Human health via consumption of drinking water		Master reference
Existing drinking water standard(s)	-	Directive 98/83/EC
Any guideline	Drinking Water Unit Risk: 9.0E-7 per µg/L Extrapolation Method — Linearized multistage procedure, extra risk Drinking Water Concentrations at Specified Risk Level: E-6 (1 in 1,000,000) = 1 µg/L	(IRIS)

5.6 Identification of issues

A **$MAC_{freshwater,\ eco}$** and an **$AA\text{-}QS_{freshwater,\ eco}$** using the species sensitivity distribution method was not yet performed. This would require the reliability of all studies and compliance with the minimum requirements of the dataset in the TGD EQS are achieved. The HC_5 values for TNT (Effects, HC_5 = 116 µg/L and No effects HC_5 = 34 µg/l), which were calculated by (Lotufo et al. 2017), indicate that the Quality standards (**$MAC_{freshwater,\ eco}$** = 1.8 µg/L and **$AA\text{-}QS_{freshwater,\ eco}$** = 0,15 µg/l) calculated with the assessment factor method are conservative values.

The data for 2-ADNT and 4-ADNT show a similar toxicity as TNT for many species. Assuming concentration additivity as a preliminary assessment, the QS for TNT can also be applied to the sum of measured TNT, 2-ADNT und 4-ADNT concentrations. However, this should be confirmed by further investigations.

Since there are great uncertainties regarding the derivation of a reliable quality standard to protect the benthic community, no value (**$AA\text{-}QS_{freshwater,\ sed.}$** or **$AA\text{-}QS_{saltwater,\ sed.}$**) has been established at this time.

5.7 Identification of any potential implementation issues in relation to the QSs derived

The QS derived serve as orientation values for the assessment of the mutational deposits in the disposal areas. The establishment of a quality standard within the framework of the Surface Water Ordinance is probably not necessary, since so far, no wide-ranging exceedance of the conservatively derived quality standard has been recorded and the pollution is essentially limited to the dumping areas of munitions.

6 Toxicological risk assessment of environmental concentrations of munition compounds

6.1 Measured concentrations of munition compounds in the Baltic and North Sea

Investigations of the UDEMM Project (UDEMM, 2019) showed that concentrations of munition compounds could be detected in water, sediment and biota in the Baltic Sea. Concentrations in water were usually low but highest in the direct vicinity of the dumped munition. Most of the water samples showed TNT concentrations on the order of 1-10 pM[9] (equals 0.000227 µg/L – 0.00227 µg/L), highlighting the critical need to use ultra-sensitive detection methods such as those developed during UDEMM. Concentrations were highest along the coast of Schleswig-Holstein, Germany. There, areas with munition dumping grounds (e.g., Kolberger Heide; Bay of Lübeck) or known munition contaminations tended to show the highest concentrations of up to 354 pM for TNT (equals 80 ng/L).

Strehse et al., 2024 investigated in the TATTOO project time trends of munition compound in mussels as well as spatial distribution in biota in the Baltic and North Seas and also the potential accumulation in the food chain. While no bioaccumulation in the food chain could be detected (Schick et al., 2022), munition compounds were clearly detected in the sediment samples from the North Sea coast of Lower Saxony. However, some of these were below the limit of quantification. The highest munition compound concentrations measured were approx. 1 ng/kg sediment. The samples came from the Jade and Jade Bay region and the island of Norderney.

All analysed fish muscle tissues/fillets and bile samples were positive for detection of munition compounds (Maser et al. 2024). In bile, the maximum concentrations of munition compounds ranged between 0.25 and 1.25 ng/ml. Interestingly, while detected TNT metabolites in the muscle tissues were in concentrations of max. 1 ng/g (dry weight), TNT itself was found in concentrations of up to 4 ng/g (dry weight). As considerable higher amounts of non-metabolized TNT were found in the fish muscle, rather than TNT metabolites, it was concluded that an additional absorption route of munition compound into fish other than per diet must exist. Maser et al. 2024 was the first study to detect munition compounds in the edible parts of fish caught randomly in the North Sea.

Annual mussel samples over 30 years received from the Federal Environmental Specimen Bank for three locations in the North and Baltic Sea were analysed for EC content (Strehse et al., 2023). Starting in 1999, initial indications of the presence of EC were obtained in the mussels from the Environmental Specimen Bank from all three locations analysed. From 2012 (Eckwarderhörne), 2013 (Königshafen) and 2017 (Darßer Ort), the first measurement signals of EC above the detection limit were detected with sufficient probability. In the samples from the 2019 and 2020 collections in the Eckwarderhörne region (Lower Saxony), 2- and 4-ADNT were also measured slightly below the limit of quantification (2-ADNT 0.14 ng/g (dry weight), 4-ADNT 0.17 ng/g (dry weight.)). It was therefore becoming apparent in all the regions analysed that munition compounds will also be detected in the mussel samples collected in the future. Due to the advancing corrosion of the metal shells of the World War munitions lying in the sea, it can be extrapolated that the munition compounds concentrations in the blue mussels (*Mytilus edulis* L.) will increase.

[9] Conversion of concentrations from pM (pmol/L) to pg/L for TNT: multiplication with 227.13 (molecular weight of TNT: 227.13 g/mol) is 227.13 pg/L or 0.22713 ng/L.

Kamman et al. (2024) analysed the flat fish dab (*Limanda limanda* L.) caught in German coastal waters of the Baltic Sea and the North Sea for contamination with munition compounds. They analysed concentrations in the bile. Probably due to rapid metabolisation, concentrations of the explosive TNT were always below limit of detection, but its metabolites 2-ADNT and 4-ADNT were detected in bile fluid up to 26.36 ng/ml and 95.91 ng/ml, respectively. Only few fish from the Baltic Sea were positive for the munition compound octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) with a maximum concentration of 0.89 ng/ml. Highest concentrations of TNT metabolites in bile fluid were detected in dab collected near the dumping site "Kolberger Heide" in the bay of Kiel (Baltic Sea). However, also dab from the North Sea were significantly contaminated with TNT metabolites.

These studies show that concentrations of TNT and its metabolites are usually low in the order of few ng/L in water, ng/kg in sediment, ng/mL in fish bile and ng/g (equals µg/kg) dry weight in muscle tissue of fish in some distance from the dumping grounds when dispersed and diluted into the marine environment.

6.2 Risk assessment of measured concentrations of munition compounds using EQS values

As concentrations alone do not inform us about any risk to the environment or human sea food consumer, standardised toxicity tests are performed for each substance. As toxicity tests are performed with few test organisms which cannot represent the biodiversity in the marine environment, safety factors are placed on top of the toxicity endpoint in order to determine a limit value. If concentrations exceed the limit value, reduction measures should be taken to reduce the environmental concentrations of the respective substance below this limit value.

For the coastal and marine environment, the EU Water Framework Directive (WFD) 2000/60/EC (European Commission, 2000) and Marine Strategy Framework Directive (MSFD) (European Commission, 2008) use this approach by defining as limit values so called Environmental Quality Standards, EQS, which are based on standardised toxicity tests and safety factors. The Environmental Quality Standards (EQS) Directive 2008/105/EC (European Commission, 2013), which was updated in 2013 (2013/39/EU), defines for a list of priority substances EQS values, which have been derived based on the procedure outlined in the EU Technical Guidance Document no. 27. These values have been included into the German Surface Waters Ordinance (Oberflächengewässerverordnung, OGewV, Anlage 8, Tabelle 2: https://www.gesetze-im-internet.de/ogewv_2016/anlage_8.html), which are used to assess the chemical status of waterbodies in the 12 nautical miles (nm) zone of coastal waters and which define EQS values for priority substances in µg/L or µg/kg wet weight.

There are two kinds of environmental quality standards: An annual average standard (AA-EQS) is set for all priority substances. For selected substances with high acute toxicity, a maximum allowable concentration (MAC-EQS) is additionally specified, which must not be exceeded in all samples. The EQS for coastal waters and seas were set using test results for marine organisms as well. If such tests are not available the inland surface water standard is just divided by 10 to derive a coastal and sea standard.

For specific pollutants that are discharged in significant quantities, the Member States must derive national environmental quality standards to protect the aquatic community. In Germany, substance volumes leading to concentrations of more than half the environmental quality standard at representative monitoring sites are defined as significant. EQS with legally binding validity have been specified for a total of 162 pollutants in the German Surface Waters Ordinance. Compliance with EQS is verified using annual averages as described by the WFD.

Munition compounds are not listed as priority substances nor as specific pollutants under the WFD. They are also until today not included in the list of substances of regional sea conventions such as HELCOM (Helsinki Commission) and OSPAR (Oslo-Paris Commission). Therefore, currently no monitoring and assessment of munition compounds is taking place within OSPAR or HELCOM. In March 2025, a proposal to include munition compounds into the Pre-CEMP monitoring programme of OSPAR was submitted to the OSPAR HASEC Committee based on work by the REMARCO project. It was approved and monitoring guidelines, quality assurance procedures and means for assessments will be developed. HELCOM just approved the inclusion of dumped munitions in the list of relevant sources of hazardous releases in Recommendation 42-43/6 for the Regional strategic approach of HELCOM´s management framework for hazardous substances (HELCOM 2025).

Considering that munition compounds have been measured in all marine matrices and that with the corrosion of the metal shells of munitions, more and more of the toxic and carcinogenic substances will be released into the marine environment, we have started developing an EQS for munition compounds. TNT is the most toxic munition compound and as its metabolites show similar toxicity as shown in our toxicity tests with the marine diatoms, we have developed draft EQS values for TNT as described in chapter 5 by following the approach of the Technical guidance document no. 27 (Schudoma et al., 2025).

When comparing the draft EQS values for TNT in water, the $MAC_{saltwater, eco}$ of 1.8 µg/L and $AA\text{-}QS_{saltwater, eco}$ of 0.15 µg/L (see chapter 5) with the measured concentrations in water of 0.01 µg/L vs. 0.08 µg/L in water in the Bay of Lübeck vs. Kolberger Heide, both EQS values are not exceeded at these munition dumping areas.

As no EQS values for TNT concentrations in sediment could be derived (see chapter 5), the highest EC concentrations of approx. 1 ng/kg sediment measured in the Jade region, cannot be judged against an EQS value. But as the toxicity of TNT-enriched sediment to marine polychaete, estuarine amphipod, freshwater midge, amphipod, oligochaete and estuarine fish occurred over a wide concentration range of 37 to 508 mg/kg (Lotufo 2017, Lotufo et al. 2021), the measured concentration in sediment is far below the measured toxic concentrations. This can be explained by the weak sorption of TNT to sediments.

No EQS value for TNT in biota to protect from secondary poisoning via the food chain could be derived as the bioaccumulation of TNT is low (see chapter 5). But two draft EQS values for TNT in biota to protect humans against adverse health effects from consuming contaminated seafood could be derived, $QS_{biota, hh}$ of 60 $\mu g/kg_{biota\ ww}$ for toxic effects of TNT and $QS_{biota, hh}$ of 4.1 $\mu g/kg_{biota\ ww}$ for carcinogenic effects of TNT (see chapter 5).

The UDEMM project showed for Kolberger Heide concentrations of 4-ADNT in mussel tissue ranging from 2.40 ± 2.13 to 7.76 ± 1.97 ng/g wet weight. As ng/g equals µg/kg, the concentrations of TNT measures in Kolberger Heide are as high as the EQS biota for carcinogenic effects of TNT. If TNT would be listed as specific pollutant according to the EU WFD and German Surface Waters Ordinance and if the EQS would be officially approved, then remediation measures would be legally required.

In the TATTOO project, TNT in fish muscle tissues was found in concentrations of up to 4 ng/g (dry weight). Assuming an average water content of 80% in fish muscle tissue, a corresponding value of 0.8 ng/g (wet weight) or 0.8 µg/kg (wet weight) can be calculated therefore below the $QS_{biota, hh}$ for carcinogenic effects. More data on TNT concentrations in fish muscle are needed to evaluate the risk for the human sea food consumer.

In the marine environment, it is well known that concentrations of hazardous substances are diluted by the huge amount of marine waters. Therefore, concentrations of munition compounds are highest at the dumping areas and should be measured there, close to the source.

6.2.1 Is TNT a PBT substance?

Annex XIII to the REACH Regulation (European Commission, 2006) sets criteria for substances that are persistent, bioaccumulative and toxic (PBT):

- A substance fulfils the persistence criterion (P) in any of the following situations:
  (a)the degradation half-life in marine water is higher than 60 days;
  (b)the degradation half-life in fresh or estuarine water is higher than 40 days;
  (c)the degradation half-life in marine sediment is higher than 180 days;
  (d)the degradation half-life in fresh or estuarine water sediment is higher than 120 days;
  (e)the degradation half-life in soil is higher than 120 days.
- A substance fulfils the bioaccumulation criterion (B) when the bioconcentration factor in aquatic species is higher than 2000.
- A substance fulfils the toxicity criterion (T) in any of the following situations: a) the long-term no-observed effect concentration (NOEC) or EC_{10} for marine or freshwater organisms is less than 0.01 mg/L; b) the substance meets the criteria for classification as carcinogenic (category 1A or 1B), germ cell mutagenic (category 1A or 1B), or toxic for reproduction (category 1A, 1B, or 2) according to Regulation EC No 1272/2008 (European Commission, 2008); c) there is other evidence of chronic toxicity, as identified by the substance meeting the criteria for classification: specific target organ toxicity after repeated exposure (STOT RE category 1 or 2) according to Regulation EC No 1272/2008.

While in rivers and lakes the toxicity of hazardous substances is most important to the ecosystem, it is the persistence and bioaccumulation in the marine environment. Marine monitoring programmes therefore measure concentrations not in water but in sediment and biota. The chemical properties of TNT are neither very persistent nor highly bioaccumulative (see chapter 5). Therefore, TNT and its metabolites are no typical substances for marine monitoring programmes. But the comparison of measured concentrations with EQS values for water and biota have shown, that for biota, the EQS value was exceeded at the investigated dumping ground. If such exceedances are confirmed by further investigations, a hot spot monitoring could be recommended beside remediation measures at such hot spots.

6.3 Conclusion and way forward

The draft EQS values for TNT derived within the CONMAR project still need to undergo a review process by the Marine Strategy Framework Directive (MSFD) expert network on contaminants and its use for assessments by OSPAR and HELCOM need to be agreed if any monitoring for munition compounds is being agreed by these regional sea conventions. At national level, the EQS for TNT could be included into the German Surface Waters Ordinance if the federal level and state organisations agree and would establish a national monitoring for munition compounds.

The comparison of the draft EQS values for TNT with measured concentration have shown that at the locations of hot spot areas of dumping grounds, the concentrations of TNT metabolites, which have a similar toxicity as TNT, can exceed the EQS value for TNT in mussels. Together with newest findings of the CONMAR measurements, this may call for remediation measures at these hot spots.

Furthermore, most hazardous substances end up in the marine environment. Beside TNT, its metabolites are nearly as toxic and carcinogenic as TNT itself. Assuming concentration additivity as a preliminary assessment, the EQS for TNT could also be applied to the sum of TNT, 2-ADNT und 4-ADNT. However, this should be confirmed by further investigations.

The mixed toxicity by the cocktail of hazardous substances and its evaluation needs further investigation. The current legislation is addressing only single substance evaluation. Toxicity tests with mixtures of munition compounds as well as other hazardous substances occurring in the respective area will be undertaken in the CONMAR-2 project by the German Environment Agency.

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