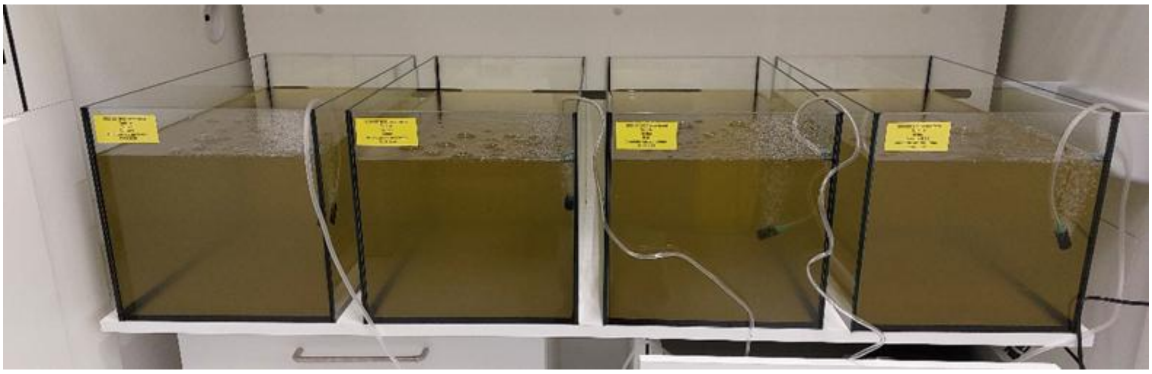
Aquarium-based assessment of NONTOX®

Testing the effectiveness and side effects in diesel-contaminated surface water



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Client	Ministerie van Infrastructuur en Waterstaat
Contact	Nur Nicanci, Fred de Haan
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Author(s)		
	Annelotte van der Linden Stefan Jansen	

Summary

Contamination of surface waters with organic pollutants poses a significant environmental concern. In this study we evaluated a commercially available bio-catalytic formulation (NONTOX®) designed to enhance degradation of petroleum hydrocarbons in surface waters by promoting the formation of micro-oxygen bubbles and stimulating biocatalytic activity.
The effectiveness in removing organic pollutants and side effects on water quality were tested the laboratory using multiple aquaria with surface water, using diesel as a model for organic pollutants at a concentration of 5 ml diesel per 1 liter of surface water.
To assess the treatment efficiency, we monitored the decrease in pollutant concentrations, with a focus on Dissolved Organic Carbon (DOC) as an indicator of organic contamination. In addition, we evaluated potential side effects and changes in chemical water quality parameters, including pH, Dissolved Oxygen (DO), and nutrient levels, to determine the impact of the treatment on the aquatic environment.
The addition of NONTOX® led to an increase in DOC, suggesting that NONTOX® enhanced the dissolution of hydrophobic diesel compounds, an essential first step in the removal process stimulated by NONTOX®. Removal of diesel did not occur initially, which might be due to nutrient limitation (N and P). This was confirmed by a decrease in DOC after targeted addition of nitrate and phosphate, suggesting degradation.
Analysis of side-effects on water quality was limited to a selective set of parameters: pH, EC, and dissolved oxygen. These parameters showed no significant differences between the aquaria. Based on these parameters and the recommendation to provide continuous aeration, no water quality issues are expected under the dosing conditions of this experiment. Further research is needed to confirm this, most notably under field conditions. Also, it should be noted that potential side-effects are strongly dependent on dosing, time, temperature, and dilution.

The presented research demonstrates effectiveness of NONTOX® to mobilize and degrade diesel, under the condition of aeration and sufficient availability of nitrate and phosphate. Side effects on EC, pH and oxygen are negligible under the conditions of these experiments. It should be noted that the these and other potential side effects should be tested under field conditions.

Further research is recommended, especially into effectiveness and side effects under field conditions and over longer timescales. An extension towards effects on microbial composition, breakdown products and other potential side effects would add to the robustness of the treatment method.

Content

1 Introduction

Contamination of surface waters with organic pollutants, including petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) and organochlorines, poses a significant environmental concern due to their persistence, toxicity and resistance to natural degradation. Addressing this issue requires innovative treatment strategies that accelerate the breakdown of these compounds through integrated physical, chemical and biological mechanisms.

In this study we evaluated a commercially available bio-catalytic formulation (NONTOX®[1]) designed to enhance degradation of petroleum hydrocarbons in surface waters by promoting the formation of micro-oxygen bubbles and stimulating biocatalytic activity.

NONTOX® consists of biodegradable fermentation products with the following key constituents and functions:

1. Surfactants which facilitate the dissolution and dispersion of hydrophobic, oil-like compounds, increasing their bioavailability for degradation.
2. Oxygen bubble stabilizers stimulating chemical and biological oxidation which can contribute to the partial breakdown of complex hydrocarbons.
3. Yeast extracts and other biological components stimulating microbial activity contributing to biological degradation of the pollutants.

Each mechanism contributes at a different stage of the treatment process: dissolution of hydrophobic compounds is anticipated to occur rapidly after application (hours to days), oxidation processes will likely proceed more gradually (days to weeks) and requires sustained aeration, while biological degradation represents the slowest phase, as it depends on adaptation of microbial communities to the introduced substrates.

To assess the treatment efficiency, we monitored the decrease in pollutant concentrations, with a focus on Dissolved Organic Carbon (DOC) as an indicator of organic contamination. In addition, we evaluated potential side effects and changes in chemical water quality parameters, including pH, Dissolved Oxygen (DO), and nutrient levels, to determine the impact of the treatment on the aquatic environment.

This study aimed to answer the following research questions:

1. How effective is NONTOX® in removing organic pollutants from surface water?
2. What side-effects may arise from its application?

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[1] **NONTOX® – Bio-Catalytic Treatment of Petroleum Hydrocarbons** NONTOX - Bio-Organic Catalyst, Inc.

2 Material and methods

2.1 Experimental set-up (aquaria)

The experiment was conducted using eight aquaria arranged in duplicate sets of four treatment conditions. Each aquarium served as a controlled mesocosm to simulate surface water environments under defined pollutant and treatment scenarios. The treatment conditions are shown in Table 2-1.

Table 2-1 Overview of aquaria aet-up and treatment conditions. Diesel was used as pollutant.

Tank	Surface water (L)	Pollutant (mL)	Bio-Catalytic (mL)	Description	Replicates
1	40	0	0	Blank control: no pollutant, no NONTOX®	2
2	40	20**	0	Pollutant only: pollutant added, no NONTOX®	2
3	40	0	6*	NONTOX® only: no pollutant, with NONTOX® dosing	2
4	40	20**	6*	Pollutant + NONTOX®: pollutant added, with NONTOX® dosing	2

* Stock solution of 100 g/L, pre-diluted with demineralized water at a 1:10 (v:v) ratio, was applied twice a week

** Assumed density of 830 kg/m³

We used glass aquaria with a total volume of 50 liters each filled with 40 liters of surface water collected from a nearby pond on the Deltares campus. The aquaria were placed under a fume hood to limit the spread of volatilized compounds and odours. To minimize evaporation, the aquaria were covered with acrylic glass lids. On day 34 of the experiment, any evaporative loss was compensated by topping up with demineralized water.

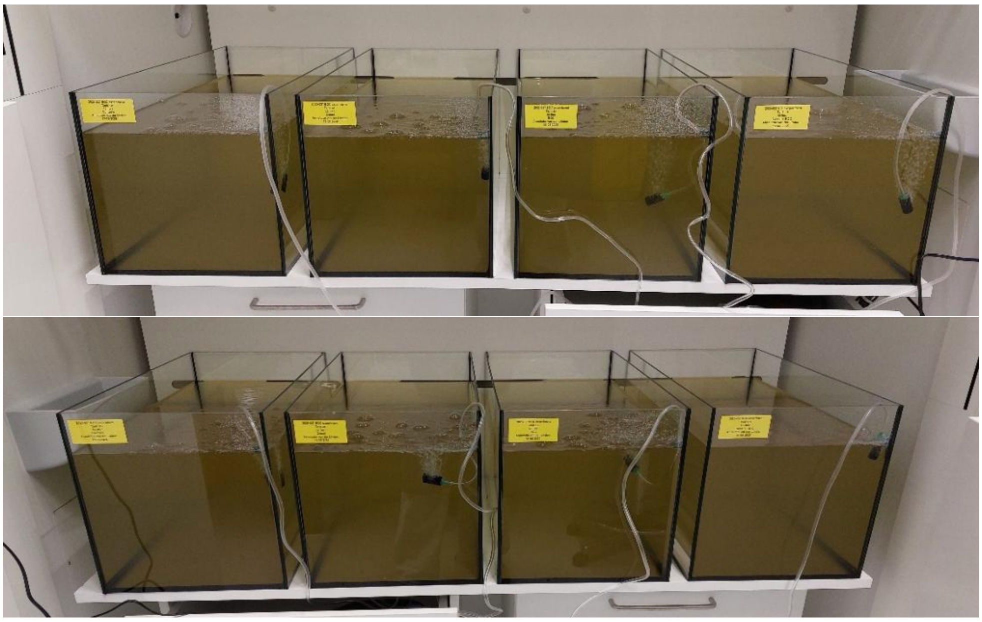

Figure 2-1 Experimental aquaria setup at the start of the study. From left to right: aquaria 1- 4, corresponding to the treatment conditions described in Table 2-1. The top panel shows the A replicates while the bottom panel shows the B replicates. The tubes are supplied with air stones and connected to an aeration pump, ensuring continuous oxygenation throughout the experiment.

All aquaria were maintained under identical environmental conditions (e.g., temperature, light exposure, aeration) to ensure comparability. To provide continuous aeration, each aquarium was equipped with an air stone attached to an aeration pump. During the first 14 days of the experiment, signs of nutrient limitation were observed, and the NONTOX® treatment did not appear to provide sufficient nutrient levels. To address this, we supplemented the aquaria with key nutrients, nitrate and phosphate, at concentrations representative of healthy natural surface waters (5 ppm NO_3-N and 0.5 ppm PO_4-P per aquarium). The experiment was conducted over a multi-week period to capture both short-term and longer-term treatment effects.

2.1.1 Pollutant selection and concentration

For this study we aimed to select a target pollutant that is a representative petroleum hydrocarbon compound in surface water contamination scenarios. Hence, we used diesel from a local pump station. The concentration applied in the aquariums was based on environmentally realistic levels observed in moderately polluted surface waters, ensuring both ecological relevance and measurable degradation potential.

2.1.2 Application of bio-catalytic agent solution (NONTOX®)

NONTOX® stock solution (10^5 mg/L) was pre-diluted at a 1:10 ratio with demineralized water and 6 mL of the resulting working solution was applied to the water surface seven times[2] during the experiment. Both the application frequency and dosing concentration were based on manufacturer guidelines and adjusted to the aquarium volume to ensure consistent exposure. Application was performed manually with a pipette while the continuous aeration via the oxygen pump ensured uniform distribution throughout the water column.

Figure 2-2 NONTOX® stock solution used for the aquarium experiment.

2.2 Monitoring pollutant and water quality parameters

Pollutant concentrations were monitored over time using DOC as a proxy for hydrocarbon contamination. In addition, chemical water quality parameters, including Electrical conductivity (EC), pH, DO, and nutrient levels (nitrate and phosphate), were measured. Sampling was conducted at regular intervals over the course of the experiment. In addition to chemical analyses, we conducted visual inspections of the aquaria to observe any physical changes or biological responses, such as change in turbidity or algal growth.

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[2] On day 0, 2, 5, 8, 14, 22, 34 since the start of the experiment.

2.2.1 Determination of pH, EC and DO

The DO concentrations, pH and EC were measured using HACH multiparameter probes. DO was determined using the Intellical LDO101 luminescent/optical sensor for dissolved oxygen (LDO) with a detection range of 0.05 – 20 mg O_2/L and an associated uncertainty of 0.1 mg/L between 0-8 mg/L and 0.2 for >8 mg/L. The pH was measured using a Intellical PHC201 Laboratory pH electrode with a range of 0 - 14 pH units and an accuracy of ±0.02 pH units. Electrical conductivity (EC) was determined using a Intellical CDC401 Laboratory conductivity probe providing a measurement range of 0.01 µS/cm - 200 mS/cm with an accuracy of 0.5% of the measured value.

2.2.2 Determination of nutrient concentrations

Anion and cation concentrations, including key nutrients such as nitrate and phosphate, were measured in the using Ion Chromatography (IC). Water samples were filtered through 0.45 µm membrane filters prior to analysis on a Dionex ICS 6000 system (Thermo Scientific). Calibration was performed using certified standard solutions from Inorganic Ventures.

2.2.3 Determination of DOC content

Dissolved organic carbon (DOC) concentrations were measured using a non-purgeable organic carbon (NPOC) analysis method. The water samples were filtered through 0.45 µm membrane filters and acidified with 1% (v/v) of 2 M Hydrochloric acid (HCl) prior to analysis on a Shimadzu TOC-L system. Calibration was performed using certified standard solutions from Reagecon Diagnostics Ltd.

3 Results and discussion

3.1 pH, EC and DO

Electrical conductivity (EC), pH and DO were monitored throughout the treatment period as shown in, respectively, Figure 3-1, Figure 3-2 and Figure 3-3.
Lines represent temporal changes across different treatment conditions.

EC shows similar patterns across all aquaria, generally increasing over time. The observed increase is likely due to evaporation (Figure 3-1). To compensate for this, the aquaria were topped with demineralized water on day 34, which caused a slight decrease of the EC in the A replicates (solid lines) and stabilization in the B replicates (dashed lines). The observed difference between replicates A and B is attributed to a change in the demineralized water source during the refill, which was necessary because of a tap malfunction in the Deltalab.

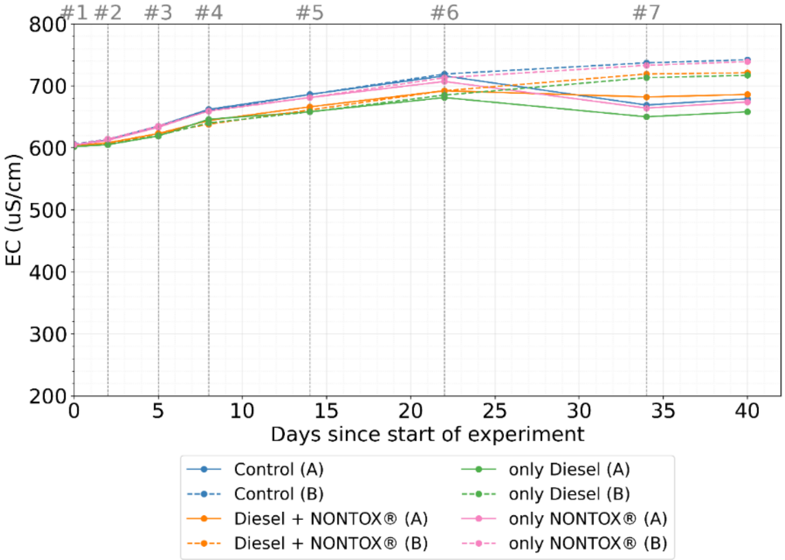

Figure 3-1 Variation in EC throughout the experiment. Color-coded lines represent different treatment conditions: (blue) untreated surface water (control); (orange) Surface water contaminated with diesel (415 mg/L) and treated with NONTOX® (15 mg/L); (green) surface water contaminated with diesel (415 mg/L); (pink) surface water treated with NONTOX® (15 mg/L).

The pH remains relatively stable throughout the experiment, ranging between 8 and 8.5 across all aquaria (Figure 3-2). A slight decrease observed around day 34 is likely due to the addition of demineralized water. Oxygen concentrations also remained consistent over time, apart from a minor drop observed around day 5 (Figure 3-3). The lack of significant differences in oxygen levels between the aquaria along with the temporal stability are likely due to the continuous aeration.

Overall, we observed no significant differences between the aquaria for any of the measured parameters, indicating that the environmental conditions remained constant throughout the experiment.

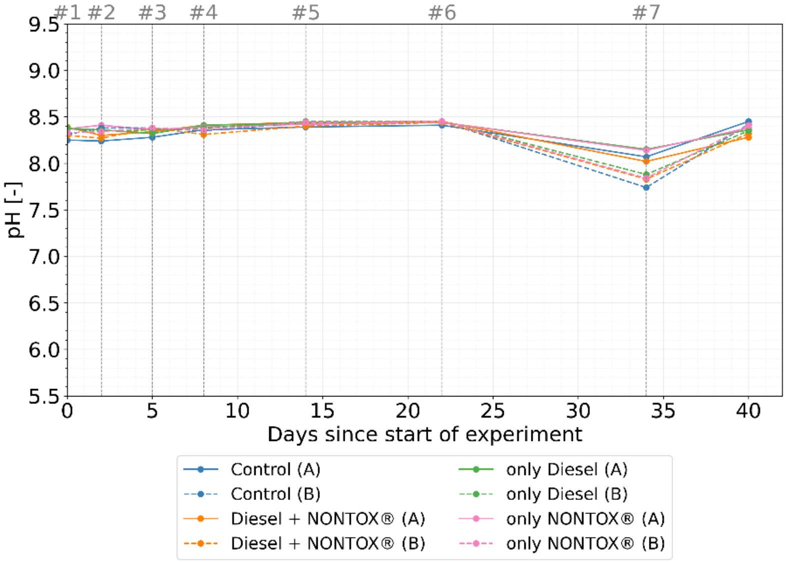

Figure 3-2 Variation in pH throughout the experiment. Color-coded lines represent different treatment conditions: (blue) untreated surface water (control); (orange) Surface water contaminated with diesel (415 mg/L) and treated with NONTOX® (15 mg/L); (green) surface water contaminated with diesel (415 mg/L); (pink) surface water treated with NONTOX® (15 mg/L).

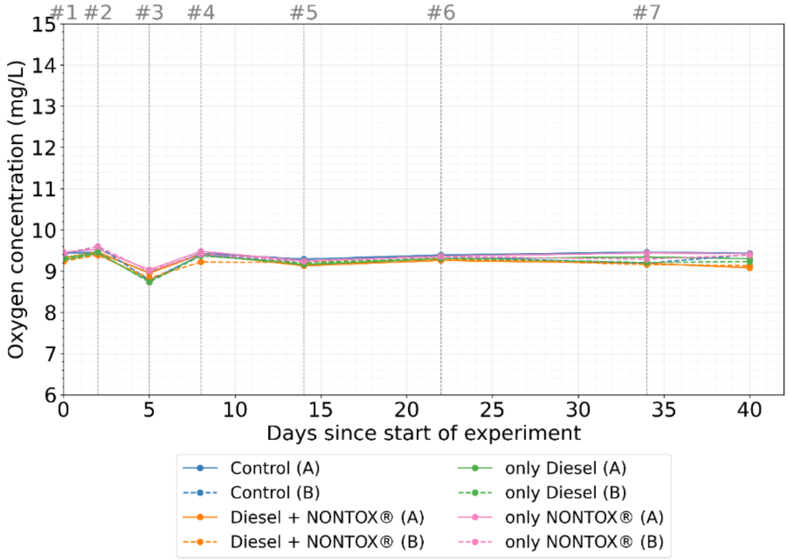

Figure 3-3 Variation in oxygen concentration throughout the experiment. Color-coded lines represent different treatment conditions: (blue) untreated surface water (control); (orange) Surface water contaminated with diesel (415 mg/L) and treated with NONTOX® (15 mg/L); (green) surface water contaminated with diesel (415 mg/L); (pink) surface water treated with NONTOX® (15 mg/L).

3.2 Nutrients

Anion and cation concentrations were measured at the start of the experiment and monitored throughout the treatment period to track the nutrient dynamics. As shown in Figure 3-4, nitrate and phosphate remained below their respective detection limits (nitrate: 0.18 mg/L;

phosphate: 0.05 mg/L) during the first 14 days of the experiment, indicating nutrient limitation. The NONTOX® treatment alone did not appear to supply sufficient nutrients levels. It may have introduced some nutrients as it contains yeast extracts, but these were probably consumed quickly. The nutrient limitation likely hindered microbial degradation. Therefore, on day 24, we spiked the aquaria with nitrate and phosphate at concentrations characteristic of healthy natural surface waters (ca. 0.5 ppm PO_4-P and 5 ppm NO_3-N per tank). The observed decline in concentration between day 35 and day 40 suggest active utilization during organic matter degradation, with a more pronounced decrease in the diesel contaminated tanks (orange and green lines) likely due to the elevated DOC levels. The exact onset of the decline could not be determined, as the nutrient concentrations were not measured between day 24 and 35.

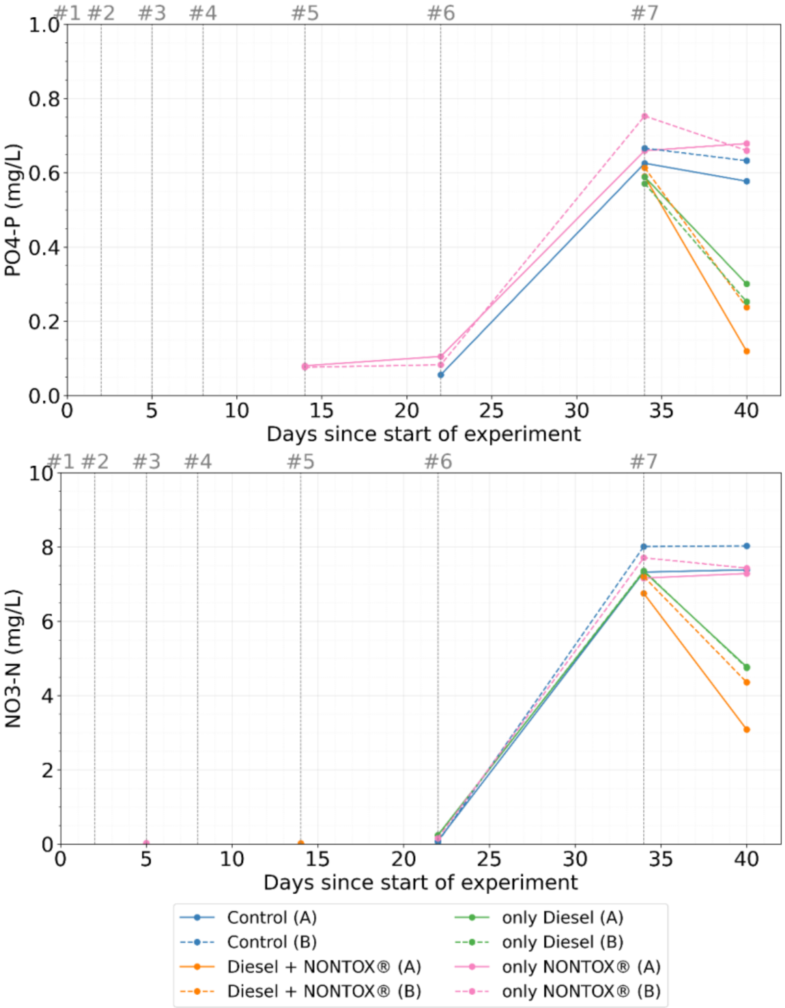

Figure 3-4 Variation in phosphate (top) and nitrate (bottom) throughout the experiment. Color-coded lines represent different treatment conditions: (blue) untreated surface water (control); (orange) Surface water contaminated with diesel (415 mg/L) and treated with NONTOX® (15 mg/L); (green) surface water contaminated with diesel (415 mg/L); (pink) surface water treated with NONTOX® (15 mg/L).

3.3 Dissolved organic carbon

Figure 3-5 shows the temporal variation in DOC concentrations throughout the experiment. DOC was used as a proxy indicator for diesel contamination. The graph shows an expected pattern: persistence in the diesel only aquaria and increased dissolution of organic pollutants followed by gradual breakdown in the treated aquaria. During the first 14 days, aquaria contaminated with diesel and treated with NONTOX® (orange lines) exhibit a notable increase in DOC concentrations. A smaller increase is also observed in the NONTOX®-treated aquaria without diesel (pink lines). In contrast, DOC levels in the control and diesel only aquaria (blue and green) remain relatively stable throughout the observation period. From day 14 onwards, the increase in DOC in the diesel + NONTOX® aquaria starts to plateau. After nutrient additions on days 22 and 34, DOC levels in these tanks begin to decline, indicating an onset of degradation.

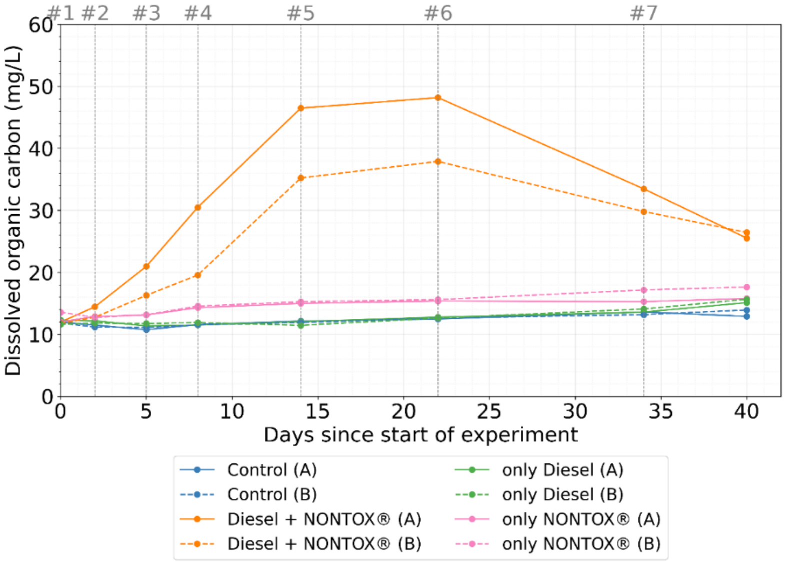

Figure 3-5 Variation in dissolved organic carbon throughout the experiment. DOC was used as a proxy indicator for diesel contamination. Color-coded lines represent different treatment conditions: (blue) untreated surface water (control); (orange) Surface water contaminated with diesel (415 mg/L) and treated with NONTOX® (15 mg/L); (green) surface water contaminated with diesel (415 mg/L); (pink) surface water treated with NONTOX® (15 mg/L).

Based on the added diesel concentration, we would expect a DOC concentration of approximately 356 mg/L if all the carbon in the diesel would dissolve. However, the DOC results indicate that at most only 13% of the diesel carbon is dissolved.

Possible reasons for this low recovery include:

- Loss of volatile components during NPOC analysis: Light hydrocarbons will escape during the acidification or purging steps of this method
- Filtration losses: The NPOC analysis requires filtration. Some diesel could have adhered to the filter
- Evaporation losses: A portion of the diesel may have volatilized from the tanks
- Incomplete dissolution: Not all diesel fully dissolved in the aqueous phase
- Adhesion to tank walls: Diesel droplets can cling to the glass surfaces
- Sequestration in biofilm: Biofilm formed on the aquarium bottom may absorb or trap diesel components

We expect that due to the possible reasons mentioned above, part of the diesel will not be retrieved in solution, but that this does not change our expectations of initial dissolution by NONTOX® followed by degradation.

3.4 Visual observations

The effects of NONTOX® treatment in the (polluted) surface water could also be observed visually. Besides foam formation after application (Figure 3-6), the different treatment conditions led to distinctive visual differences in turbidity between the aquaria.

Figure 3-6 Example of the foam formation after NONTOX® treatment to aquarium 4B as generally observed.

At the start of the experiment the diesel contaminated aquaria (2A/B and 4A/B) showed an evident floating diesel layer, which was absent in the other aquaria (1A/B and 3A/B) (Figure 3-7).

Two days after the first application of NONTOX®, the NONTOX®-treated diesel aquaria (4A and B) still showed a floating diesel layer, while emulsified oil accumulation started to appear in the corners of non-treated diesel aquaria (2A and B, Figure 3-8). We did not observe a noticeable difference in odour at this stage.

Figure 3-9 shows the visual difference after approximately a week. By then we started to observe clear distinctions: the unpolluted aquaria (1A/B and 3A/B) remained clear, while non-treated diesel aquaria showed emulsified oil and strong petroleum odours. In contrast, NONTOX®-treated diesel aquaria showed signs of diesel dissolution (higher turbidity) and reduced smell, consistent with DOC measurements. After 30 days, we observed increased turbidity and biofilm formation on the bottom and walls of NONTOX®-treated diesel aquaria (Figure 3-10). The unpolluted aquaria, both the control and NONTOX® only, did not show any visual changes in turbidity throughout the experiment.

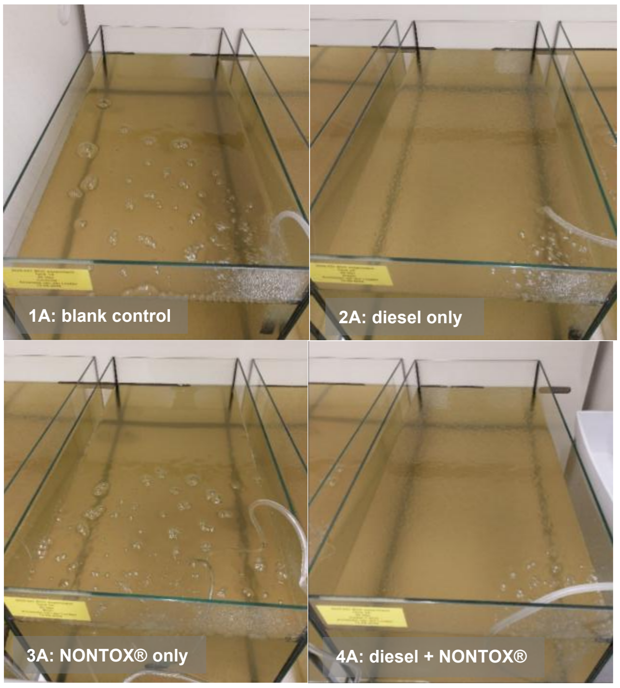

Figure 3-7 Initial setup of aquaria at the start of the experiment, following the addition of diesel to aquaria 2 and 4. Displayed here are the A replicates in clockwise order starting from the top left corner as follows: (1) blank control; (2) diesel pollutant only, (3) NONTOX® treatment only, (4) diesel pollutant and NONTOX® treatment. Note the floating diesel film at the water surface in aquaria 2 and 4.

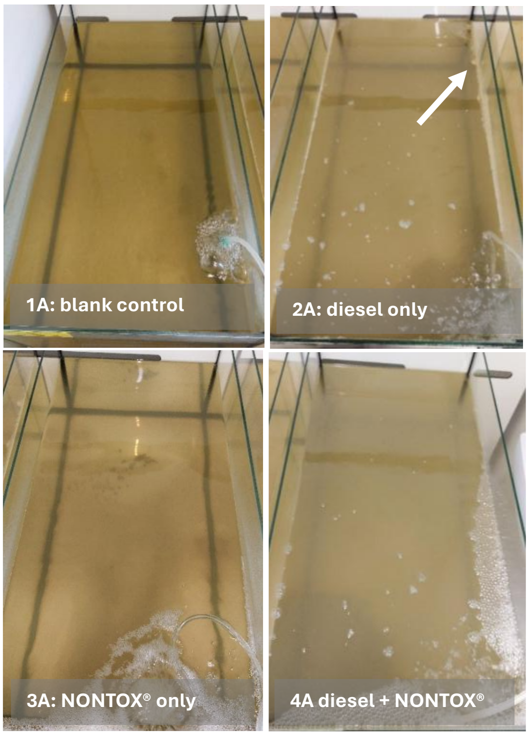

Figure 3-8 Visual differences between the aquaria after day 2 of the experiment. Displayed here are the A replicates in clockwise order starting from the top left corner as follows: (1) blank control; (2) diesel pollutant only, (3) NONTOX® treatment only, (4) diesel pollutant and NONTOX® treatment. Arrow indicates emulsified oil accumulation in aquaria 2.

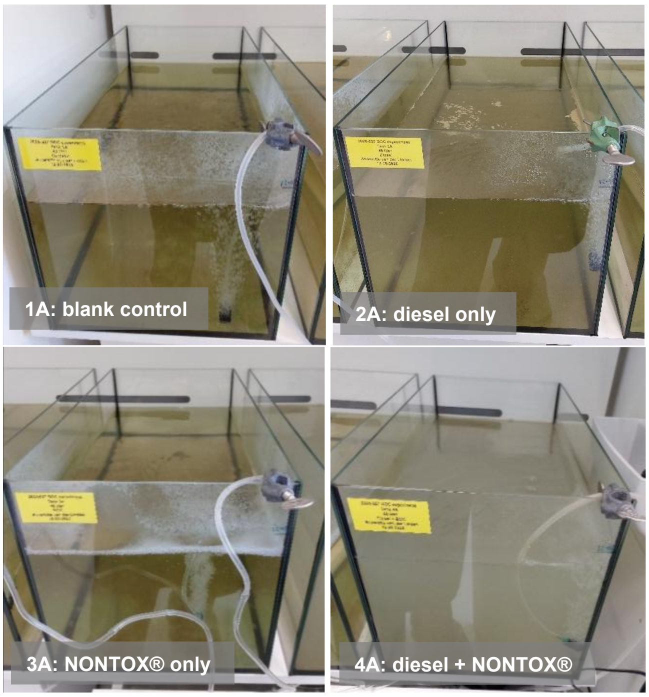

Figure 3-9 Visual differences between the aquaria after one week. Displayed here are the A replicates in clockwise order starting from the top left corner as follows: (1) blank control; (2) diesel pollutant only, (3) NONTOX® treatment only, (4) diesel pollutant and NONTOX® treatment. Note the emulsified oil in 2A and higher turbidity in 4A.

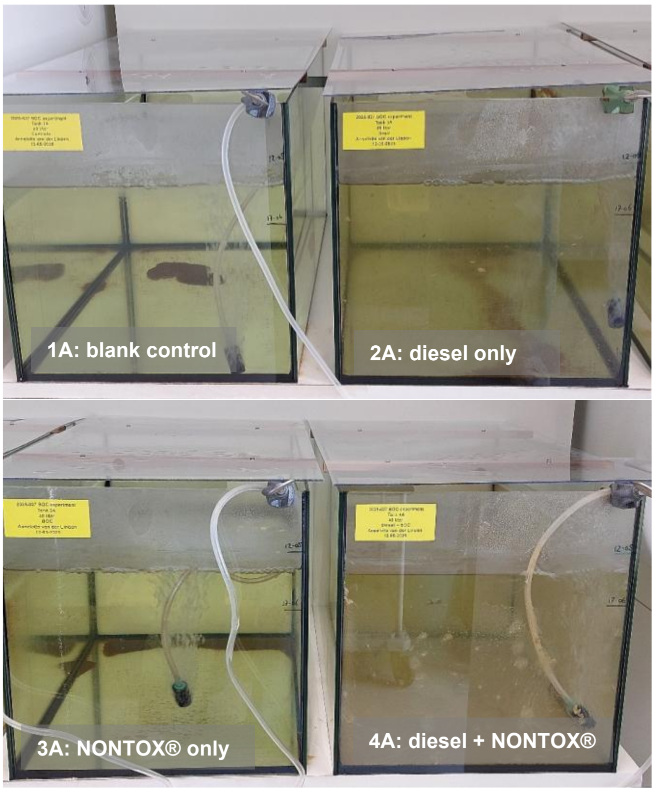

Figure 3-10 Visual differences between the aquaria after 30 days. Displayed here are the A replicates in clockwise order starting from the top left corner as follows: (1) blank control; (2) diesel pollutant only, (3) NONTOX® treatment only, (4) diesel pollutant and NONTOX® treatment.

4 Conclusions and recommendations

In this study we analysed the effectiveness of NONTOX® in removing diesel from surface water. The experimental results showed that the addition of NONTOX® led to an increase in DOC, suggesting that NONTOX® enhanced the dissolution of hydrophobic diesel compounds. It is likely that nutrient limitation during the initial weeks hindered subsequent microbial degradation. Although NONTOX® may have introduced some nutrients, these were probably consumed rapidly. These findings highlight the importance of assessing baseline nutrient availability prior to application, as NONTOX® alone may not sustain microbial activity under nutrient-limited conditions. After targeted addition of nitrate and phosphate, a decrease in DOC was observed in the diesel-contaminated aquaria treated with NONTOX®, suggesting degradation. Although the application of NONTOX® caused foam formation, we do not have data showing that the bubbles directly attribute to increased degradation. Analysis of side-effects on water quality was limited to a selective set of parameters: pH, EC, and dissolved oxygen. These parameters showed no significant differences between the aquaria. Based on these parameters and the recommendation to provide continuous aeration, no water quality issues are expected under the dosing conditions of this experiment. Further research is needed to confirm this, most notably under field conditions. Also, it should be noted that potential side-effects are strongly dependent on dosing, time, temperature, and dilution.

We did not directly analyse the microbial composition, but it is possible that microbial community shifts occurred during the experiment. In aquaria containing bioavailable diesel components, oil-degrading bacteria are likely to thrive, potentially outcompeting other species.
The microbial degradation of organic pollutants might lead to unknown intermediate compounds, as the breakdown pathways are unclear. Some of these intermediate compounds could be toxic.
Additionally, biodegradation processes consume substantial amounts of oxygen, and inadequate aeration may result in hypoxic or anoxic conditions, further influencing microbial dynamics and pollutant breakdown, and potentially affecting aquatic live.
The experiment was conducted at a stable temperature of approximately 17 °C. However, the temperature sensitivity of the biodegradation was not assessed. The behaviour of both diesel and microbial communities under higher or lower temperature conditions remains uncertain.
To better understand the ecological and biochemical implications and the effectiveness in removing other organic pollutants, further research is recommended. Future studies should focus on:

- Microbial community composition and dynamics under varying environmental conditions
- Detailed characterization of degradation pathways and identification of intermediate products and their toxicity
- The impact of aeration efficiency on oxygen availability
- Temperature-dependent effects on the degradation rates
- Effectiveness in removing other pollutants
- The effects of dosing and environmental conditions under field conditions (e.g. dosing concentrations, dilution, etc).

Deltares is een onafhankelijk kennisinstituut voor toegepast onderzoek op het gebied van water en ondergrond. Wereldwijd werken we aan slimme oplossingen voor mens, milieu en maatschappij.

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