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# The Synergy between Herbicides, Oil and Solvents in Bio-Toxicity to *Phaeodactylum tricornutum* Bohlin-Implications for Interpretation of Field Sample Data

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## Abstract

Bioassays of the herbicides Simazine and Atrazine, alone and as cocktails with oil and solvents were performed by a modified ISO 10253 1995 bio-toxicity test, with the test diatom *Phaeodactylum tricornutum* Bohlin. This laboratory bioassay was necessary to interpret pollutant data from field studies in the Northern Black Sea.

The present study establishes the sensitivity of the test organism to these pollutants in different combinations. Cultures of the test alga were exposed to dilution series of ethanol, DMSO, oil, Atrazine and Simazine. Ethanol was toxic to the diatoms at concentrations 100 times below DMSO, which was therefore used in the following tests, in a safe concentration (0.03% v/v).

The oil (up to 10% v/v) alone was not significantly toxic, but with DMSO it was moderately toxic at concentrations ≥ 0.032% (v/v). The algal growth after 2-4 days was significantly reduced by ≥ 0.1 mg $L^{-1}$ of Atrazine or Simazine in 0.03% v/v DMSO. The presence of oil and DMSO increased herbicide toxicity to the algae. The effective concentrations for the bio-test are far above the ecologically relevant concentrations of the pollutants found in most natural waters. The data suggest, however, that in the interpretation of algal bioassays of field water samples, the impact of synergy between different pollutants in pollutant cocktails should be considered.

**Keywords:** Marine pollution; Bioassay; Bio-test; The Black Sea; Pollution-cocktail effect

**Abbreviation:** DMSO: Dimethyl Sulfoxide; hrs: Hours; d: Days; ESW: Enriched Seawater; RSE: Relative Standard Error

## Introduction

The prevalent pollution by herbicides can damage coastal ecosystems, especially phytoplankton, and as a logical consequence phytoplankton bio-tests for the relevant pollutants have been developed [1]. Similar pollution by hydrocarbons (with reports as early as Lincoln) [2] and organic solvents (Hutchinson) [3] are prevalent and worrisome in their possible impacts on aquatic organisms but their specific impact on the phytoplankton, especially together (cocktails) has been less often studied [4,5].

When it was, DMSO was found to be far less toxic to microalgae than ethanol and methanol [6] and to increase the toxicity of Atrazine [7]. An EU-funded survey of the northern Black Sea (INCO COPERNICUS PROJECT IC 15CT 96 0105, data not shown), considered a polluted sea [8], identified and measured the concentration of several pollutants, which included the herbicides Atrazine and Simazine (a total of below 100 ng $L^{-1}$), and various hydrocarbons (a total average below 10 mg $L^{-1}$, i.e., 0.011% v/v).

Cocktail effects and synergistic interactions of chemicals in mixtures are relevant to both the public and the regulatory authorities, since some chemicals can enhance (synergism) or diminish (antagonism) the effect of other chemicals on the ecosystem [9-11]. For instance, dispersants, when they are added to crude oil, increase its solubility and bioavailability, and thereby its possible impact on the marine ecosystem [12].

Evaluating the overall response of organism to particular components in mixtures of pollutants requires careful experiments. An evaluation by one of us of the impact of river pollution on several aquatic macrophytes emphasized the importance of studying the cocktail effects of the relevant pollutants, rather than one pollutant at a time [13]. Furthermore, they presented the dilemma in the selection of the target bioindicator, between overly sensitive and overly resistant species.

The present study evaluates the toxicity of herbicides, oil and solvents and interactions therein, by a modified standard bio-test [14,15] with the diatom *Phaeodactylum tricornutum* Bohlin, which was a suitable species with an intermediate level of sensitivity [16].

## Materials and Methods

Cultures of the marine diatom *Phaeodactylum tricornutum* strain Cough [15], after being made axenic [17], were exposed to concentration series of the tested chemicals separately and in cocktails, following the international protocol for algal growth inhibition, modified from the Marine 72 hrs EC50 Algal Growth Inhibition Study, ISO 10253, 1995 which had been designed specifically for toxicity tests to seawater microalgae.

Due to the high variability in the data, and the death of some treatments before 72 hrs, we modified the data analysis.

The axenic cultures were maintained in a 24˚C (± 1) culture room under four fluorescence bulbs (Osram L 36 W/10, ~80 µmol photons $m^{-2}$ $s^{-1}$), suspended above a large custom-made variable-speed shaker (Ari J. Levi, Israel), which could hold several dozens 250 ml Erlenmeyer flasks, each containing 100 ml of enriched seawater (ESW) culture medium.

Shaker speed was adjusted to provide an optimal water movement. The medium was modified from ISO 10253 [14]. Clean Red-Sea seawater was diluted to a salinity of 30 mg $kg^{-1}$ and then nutrient-enriched to the final concentrations in a liter of the medium (Table 1).

| | Nutrient | Final medium concentration $L^{-1}$ |
|---|---|---|
| 1 | $FeCl_3$-$6H_20$ | 150 µg (Fe) |
| 2 | $MnCl_2$-$4H_2O$ | 605 µg (Mn) |
| 3 | $ZnSO_4$-$7H_2O$ | 150 µg (Zn) |
| 4 | $CuSO_4$-$5H_2O$ | 0.6 µg (Cu) |
| 5 | $CoCl_2$-$6H_2O$ | 1.5 µg (Co) |
| 6 | $H_3BO_3$ | 17 µg |
| 7 | $Na_2EDTA$ | 15 µg |
| 8 | Thiamin hydrochloride | 25 µg |
| 9 | Biotin | 0.005 µg |
| 10 | Vitamine $B_{12}$ | 0.05 µg |
| 11 | $K_3PO_4$ | 3 mg |
| 12 | $NaNO_3$ | 50 mg |
| 13 | $Na_2 SiO_3$-$5H_2O$ | 15 mg |

**Table 1:** Final concentrations of nutrients in a final liter of 30 mg $kg^{-1}$ ESW medium, as used in the culture of the diatom (modified from ISO 10253, 1995).

Several batches of 1.25 L stock culture of the diatom were pre-grown under batch conditions for each experiment in 3 L, 17 cm diameter Fernbach flasks, placed on a shaker. While the cultures were still growing exponentially, 10 ml of it was transferred to each experimental 250 ml Erlenmeyer flasks and diluted by fresh medium to 100 ml.

From here the procedure was in principal as graphically described in Katsumata et al. [18]. The diluted suspensions of the diatom (usually 5-10 × $10^6$ cells $ml^{-1}$, OD 674 of 0.06) were grown one day for acclimation and then exposed under batch conditions in triplicates to dilution series of the tested chemicals and cocktails, with the controls being flask triplicates of algae with zero concentration of the tested pollutants, blanks being flask triplicates of the medium and the tested chemicals without the algae.

The pollutant solutions were added to the flasks at time zero of each experiment, vigorously hand shaken and placed randomly on the shaker table.

The optical density values of the cultures after the subtraction of the blanks were plotted (Prism 8, GraphPad Software, San Diego, California) and compared. The impacts of the chemicals were tested individually and in mixtures, in the concentration ranges that were effective in preliminary individual tests.

The algal concentration in each flask was measured spectro-photometrically at 24 hrs intervals, after the establishment of linearity between optical density in red light (674 nm), using the Shibata method [19] and modified from [15] (Equation 1). Briefly, a known volume of the culture was filtered onto a 2.4 cm GF/C filter, which was then cut to size and placed inside a 1 cm cuvette.

This was placed in a double-beam spectrophotometer (Kontron Uvikon 720, Kontron-Instruments, Neufahrn, Germany) cuvette holder close to the phototube, with the algae side facing onto the light beam. This allowed for a large fraction of the light that hit the filter to scatter into the phototube. A cuvette with a clean wet (by clean medium) filter was placed in the reference beam.

$$Y = 12,176,381 * X - 682,123 \quad \text{(Equation 1)}$$

($r^2$=0.97; P<0.0001), Where Y: cells $ml^{-1}$ and X: *in vivo* absorption units (OD 674 nm)

95% confidence interval of the slope (of X): 11,245,155 to 13,072,632 cells $ml^{-1}$ OD $unit^{-1}$. The chemicals tested were Atrazine, Simazine, pure oil (Sonol 560 automobile hydraulic mineral oil, which is readily available at high purity), ethanol, methanol and DMSO (Table 2).

| | Chemical | Final medium concentration |
|---|---|---|
| 1 | Methanol, % v/v | 0, 0.002, 0.01, 0.1, 1 |
| 2 | Ethanol, % v/v | 0, 0.002, 0.01, 0.1, 1 |
| 3 | DMSO, mg $L^{-1}$ | 0, 0.002, 0.01, 0.1, 1 |
| 4 | Hydraulic oil, % v/v | 0, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10 |
| 5 | Atrazine, mg $L^{-1}$ | 0, 0.01, 0.032, 0.1, 0.32, 1, 3.2, 10 |
| 6 | Simazine, mg $L^{-1}$ | 0, 0.0032, 0.01, 0.032, 0.1, 0.32, 1, 3.2 |

**Table 2:** Concentrations of the chemicals tested in the toxicity tests with *Phaeodactylum tricornutum*. The range for each chemical was established in a preliminary test. DMSO as a solvent of other pollutants was always of 0.03% final concentration.

## Measures of Growth

Growth rates varied between the mathematical methods used in the literature, and therefore we assessed the toxicity by three approaches (Tables 3-8).

Growth curves were calculated by Prism 8 according to the following models:

- An exponential growth model

$$Y = Y_0 * exp(k * x) \quad \text{(Equation 2)}$$

Where $Y_0$ is the starting absorption (OD 674 nm), Y is the absorption at any given time (X), k is the rate constant ($hrs^{-1}$), and doubling time (the time needed for Y to double) is calculated as ln (2)/k.

- Log Y vs linear X

$$Y = 10^{(slope * X + Yintercept)} \quad \text{(Equation 3)}$$

Where Y is OD 674 nm at time X (hrs), the slope is the log of the change in Y. Due to the large variability of the data, especially in the first hrs in each experiment, and the inconsistent duration due to the early diatom death in some treatments, the difference in algal density (measured as OD 674 nm) of each treatment from the control data on

the last day of each experiment was also examined by a t-test (Table 3-8).

## Pollutant Preparation

Herbicides stocks were prepared in a 20% (v/v) DMSO/water at a concentration of 2000 mg $L^{-1}$. This stock solution was diluted as necessary with the same 20% DMSO solution, and then each 100 ml flask received 156 µl of the solution, resulting in a final solvent concentration of 0.03% v/v of the culture.

## Results

As recommended by the ISO 10253 (1995) method, the pH did not vary by more than 1 unit during each entire test (data not shown).

## Solvents

Preliminary tests (not shown) identified the concentration ranges that had an impact on the algae, i.e., that were between complete and undetectable toxicities.

Further tests showed that the solvent methanol (data not shown) and ethanol (Figure 1a and 1b) were more toxic than DMSO to the algae at the range tested (up to 1%) (Table 3).

Significant growth inhibition with ethanol began at a concentration of between 0.01% and 0.1% (Table 3). The culture doubling time increased by up to 38% over the control (at 0.01% ethanol) and the logarithmic growth slope and the final cell density decreased by up to ca 60% and 65%, respectively (Table 3).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Pollutant (Concentration)** | **Doubling time, estimated by the exponential growth model (Equation 2)** | **Growth curve slope, based on the log-linear growth model (equation 3)** | **Final sample average as % of control (RSE), t-test, Δ from control p-value,** | **2-W ANOVA results** |
| Units | | Hrs × generation$^{-1}$ | log OD hrs$^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |
| 1. | **Ethanol** | | | | Time p<0.0001 [64]; Concentration p<0.099 [3.9]; Interaction p<0.0001 [11.5] |
| 1.1. | 0 | 20.12 | 0.01496 /100 | 100 (3.5) | |
| 1.2. | 0.002 | 20.24 | 0.01487/99 | 94 (4.9), 0.77 | |
| 1.3. | 0.01 | 46.6 | 0.006465/43 | 45 (25), 0.005 * | |
| 1.4. | 0.1 | 27.72 | 0.01086/73 | 66 (17.2), 0.046 * | |
| 1.5 | 1 | 26.59 | 0.01132/76 | 63 (3.8), 0.011 * | |
| 2. | **DMSO** | | | | Time p<0.0001 [91]; Concentration p=0.1688 [0.6]; Interaction p=0.014 [1] |
| 2.1. | 0 | 21.48 | 0.01402/100 | 100 (2.7) | |
| 2.2. | 0.002 | 22.44 | 0.01342/96 | 101 (11.6), 0.99 | |
| 2.3. | 0.01 | 20.13 | 0.01495/107 | 98 (8.0), 0.78 | |
| 2.4. | 0.1 | 22.69 | 0.01326/95 | 85 (6.3), 0.10 | |
| 2.5. | 1 | 24.55 | 0.01226/87 | 79 (4.9), 0.018 * | |

**Table 3:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of the ethanol and DMSO experiments, as a percent of the control, from a bio-toxicity test with cultures of the diatom *Phaeodactylum tricornutum*. Concentration units: Solvents and oil- % v/v; Herbicides- mg $L^{-1}$. *: significant at p<0.05.

With DMSO, only the 1% concentration significantly inhibited the algal growth (Figure 1c and 1d; Table 3). Doubling time at the 1% DMSO concentration increased over the control by merely 14%, while the logarithmic growth slope and the final cell density were below the respective control values by up to about 13% and 21%, respectively (Table 3). DMSO was therefore selected to dissolve the herbicides and the oil.

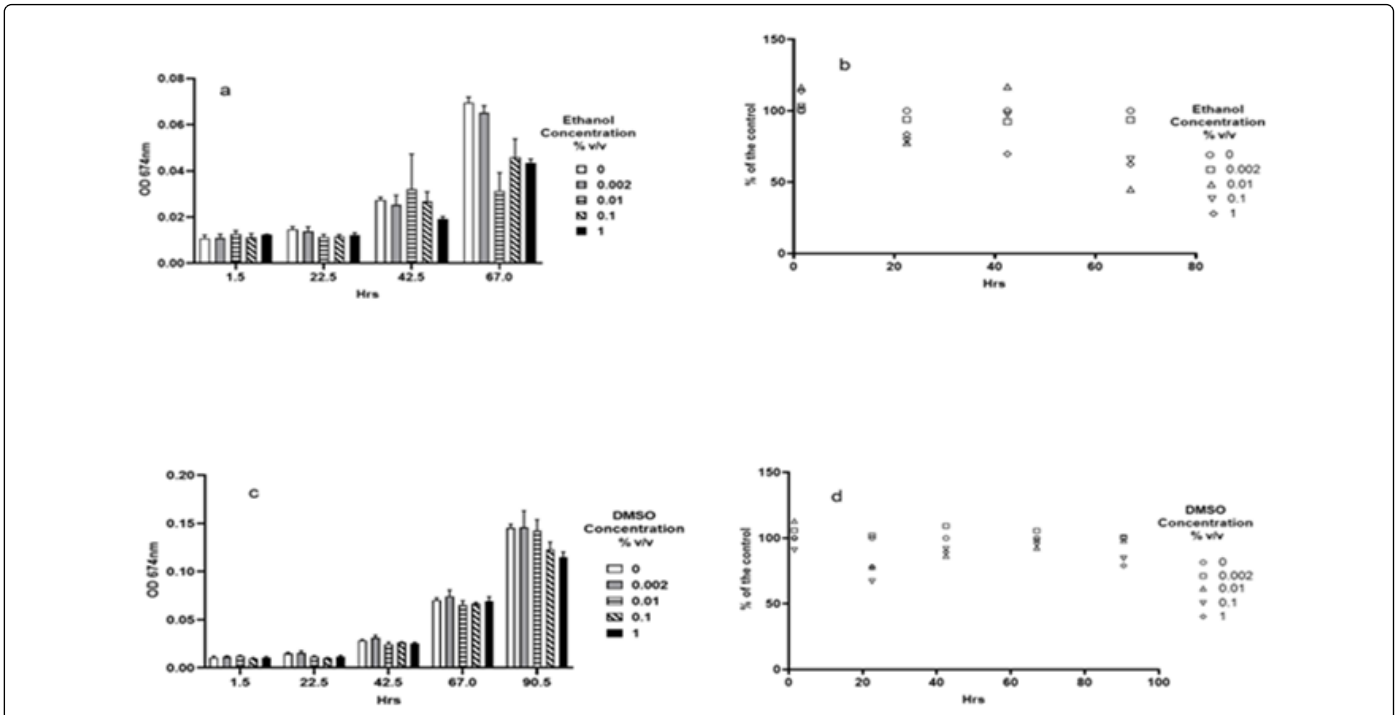


**Figure 1:** Algal (*Phaeodactylum tricornutum*) density in optical density units and as a percent of control (no solvent) when grown with ethanol (a and b, respectively) and DMSO (c and d, respectively).

## Hydraulic oil

The impact of oil on the growth of the algae was examined first, by an oil dilution series in emulsions with the culture, without solvent (Figure 2).

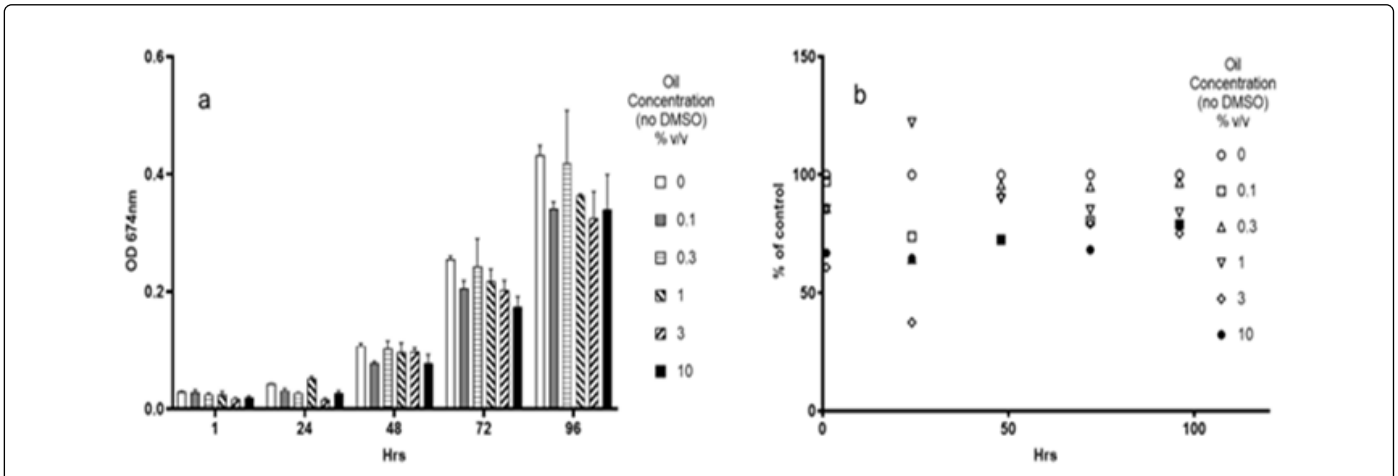


**Figure 2:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units and as a percent of control (no oil) with a concentration series of hydraulic oil (a and b, respectively).

The 2-W ANOVA of the entire experiment showed no impact of the oil concentration on the overall variability in cell density, and similarly no significant drop in growth by any of the three growth measures, but the impacts of time and of the interaction (Time × Concentration) were highly significant (Table 4).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Pollutant (Concentration)** | **Doubling time, estimated by the exponential growth model (Equation 2)** | **Growth curve slope, based on the log-linear growth model (equation 3)** | **Final sample average as % of control (RSE), t-test, Δ from control p-value** | **2-W ANOVA results** |
| Units | | Hrs × generation-1 | log OD $hrs^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |
| 1. | **Oil, no DMSO** | | | | Time p<0.0001 [88]; Concentration p=0.1363 [1.6]; Interaction p=0.6355 [<1] |
| 1.1. | 0 | 25.24 | 0.01193/100 | 100 (3.9) | |
| 1.2. | 0.1 | 25.07 | 0.01201/101 | 79 (3.5), 0.038 * | |
| 1.3. | 0.3 | 24.24 | 0.01242/104 | 97 (21.5), 0.98 | |
| 1.4. | 1 | 26.77 | 0.01125/94 | 84 (0.8), 0.09 | |
| 1.5. | 3 | 25.85 | 0.01164/98 | 75 (13.9), 0.106 | |
| 1.6. | 10 | 22.77 | 0.01322/111 | 79 (17.3), 0.15 | |
| 2. | **Oil in 0.03% DMSO** | | | | Time p<0.0001 [95]; Concentration p<0.0001[2]; Interaction p<0.0001 [2] |
| 2.1. | 0 | 15.2 | 0.0198/100 | 100 (3.0) | |
| 2.2. | 0.01 | 15.4 | 0.01955/99 | 90 (2.0), 0.1073 | |
| 2.3. | 0.032 | 16.65 | 0.01808/91 | 79 (5.6),0.048 * | |
| 2.4. | 0.1 | 15.63 | 0.01926/97 | 75 (8.1), 0.045 * | |
| 2.5. | 0.32 | 13.96 | 0.02157/109 | 99 (3.1), 0.8535 | |
| 2.6. | 1 | 15.49 | 0.01943/98 | 79 (4.6), 0.034 * | |
| 2.7. | 3.2 | 17.01 | 0.0177/89 | 72 (4.3), 0.009 * | |
| 2.8. | 10 | 16.31 | 0.01846/93 | 79 (1.1), 0.0075 * | |

**Table 4:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of two oil experiments. Details in the legend to Table 3. *: significant at p<0.05.

However, in individual comparisons of the treatments to the control the impact of the oil on the growth of the diatom was usually significant at first, but not after 96 hrs (t-tests, p<0.05, data not shown).

This decrease in impact is evident in the time-course of density as a percentage of the control (Figure 2b). The presence of DMSO made the oil toxic to the diatom starting at a concentration of 0.032% (v/v) (Figure 3), so that in the ANOVA, concentration, time and the interaction between them were all highly significant.

However, the impact of oil in DMSO on the individual growth parameters of doubling time, logarithmic growth curve and the final cell density was small, with maximal impacts of +12%, -11% and –28% respectively (Table 4). Still, in five of the treatments, the final cell density dropped significantly below the control by oil in DMSO (Figure 3, Table 4).

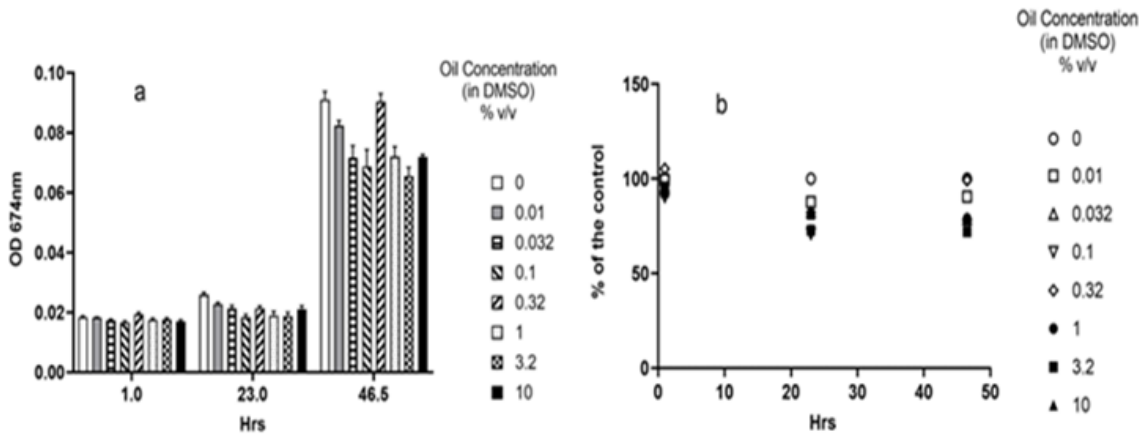


**Figure 3:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units and as a percent of control (no oil) with a concentration series of hydraulic oil with 0.03% DMSO (a and b, respectively).

## Atrazine

Atrazine in DMSO inhibited the algae increasingly with time, right from 1 hr after the onset of the experiment (Figure 4). The data varied more at first when the absolute OD values were low, but with time the inhibition became apparent and increased in correlation with herbicide concentration (Figure 4, Table 5).

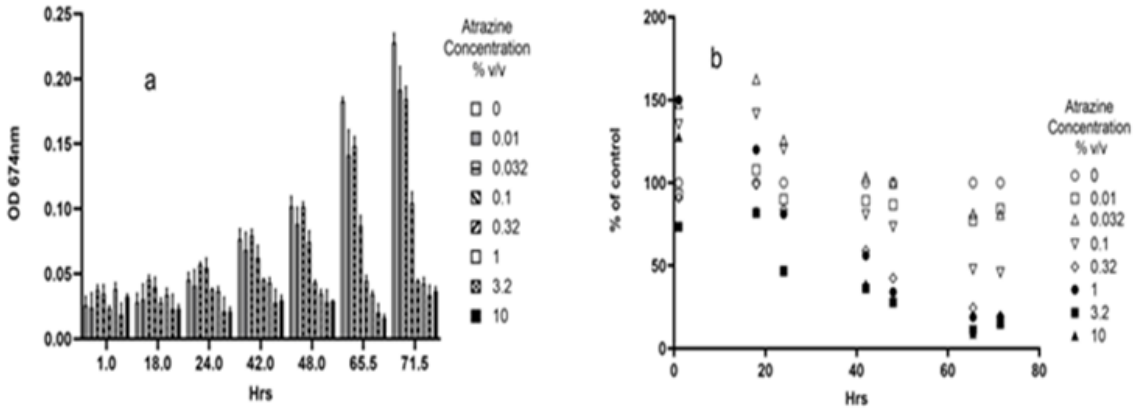


**Figure 4:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units and as a percent of control (no Atrazine) with a concentration series of Atrazine in 0.03% DMSO final concentration (a and b, respectively).

The concentration of 0.1 mg $L^{-1}$ of Atrazine in DMSO was critical. Doubling time doubled, and the logarithmic growth rate and final cell density dropped below 50% of the control at that concentration, so that the higher Atrazine concentrations led to nearly total inhibition after 65.5 and 71.5 hrs (Figure 4, Table 5).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Pollutant (Concentration)** | **Doubling time, estimated by the exponential growth model (Equation 2)** | **Growth curve slope, based on the log-linear growth model (equation 3)** | **Final sample average as % of control (RSE), t-test, Δ from control p-value.** | **2-W ANOVA results** |
| Units | | Hrs × generation$^{-1}$ | log OD hrs$^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |

| | | | | | |
|---|---|---|---|---|---|
| 1. | Atrazine in 0.03 DMSO (mg $L^{-1}$) | | | | Time p<0.0001 [30]; Concentration p<0.0001 [33]; Interaction p<0.0001 [32] |
| 1.1. | 0 | 19.67 | 0.01531/100 | 100 (3.1) | |
| 1.2. | 0.01 | 21.22 | 0.01418/93 | 84 (9.3), 0.5679 | |
| 1.3. | 0.032 | 27.85 | 0.01081/71 | 81 (5.1), 0.1201 | |
| 1.4. | 0.1 | 45.52 | 0.006614/43 | 46 (8.0), 0.0021 * | |
| 1.5. | 0.32 | 90.85 | 0.003313/22 | 19 (2.3), <0.0001 * | |
| 1.6. | 1 | 876.1 | 0.0003436/2.2 | 19 (11.1), 0.0002 * | |
| 1.7. | 3.2 | 131.2 | 0.002294/15 | 14 (21.6), 0.0003 * | |
| 1.8. | 10 | 5227 | 0.00005759/0.4 | 16 (8.1), <0.0001 * | |
| 2. | Simazine in 0.03% DMSO (mg $L^{-1}$) | | | | Time p<0.0001 [55]; Concentration p<0.0001 [23]; Interaction p<0.0001 [21] |
| 2.1. | 0 | 15.72 | 0.01915/100 | 100 (2.0) | |
| 2.2. | 0.0032 | 15.83 | 0.01902/99 | 94 (5.5), 0.678 | |
| 2.3. | 0.01 | 15.21 | 0.01979/103 | 98 (3.6), 0.891 | |
| 2.4. | 0.032 | 14.7 | 0.02048/107 | 98 (3.1), 0.607 | |
| 2.5. | 0.1 | 14.88 | 0.02024/106 | 69 (7.6), 0.010 * | |
| 2.6. | 0.32 | 15.54 | 0.01938/101 | 32 (2.6), <0.001 * | |
| 2.7. | 1 | 21.78 | 0.01382/72 | 12 (7.5), <0.001 * | |
| 2.8. | 3.2 | 33.35 | 0.009/27 | 5 (25.7), <0.001 * | |

**Table 5:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of the herbicide experiments. Details in the legend to Table 3. *: significant at $p<0.05$.

## Simazine

Inhibition of the diatom by Simazine in DMSO was strong already after a day and led to the death of the diatoms in the highest pollutant levels after 53.5 hrs (Figure 5). Doubling time doubled and the logarithmic growth rate halved only at the highest concentration examined (3.2 mg $L^{-1}$ of Simazine in DMSO), but the final cell density fell significantly below the control at the four pollutant concentrations ≥ 0.1 mg $L^{-1}$ of Simazine in DMSO, and the higher concentrations led to total inhibition and cell death after 53.5 hrs (Figure 5, Table 5).

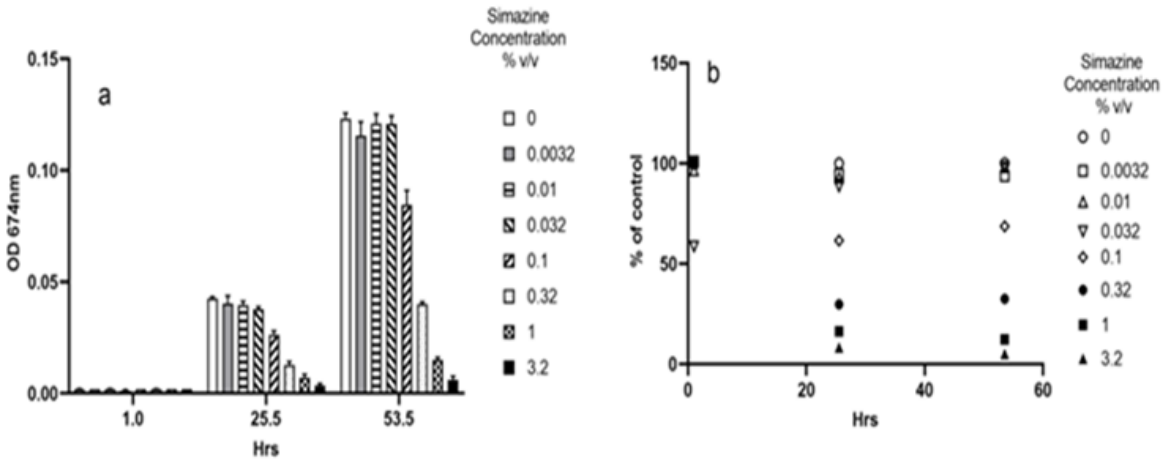


**Figure 5:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units and as a percent of control (no Simazine) with a concentration series of Simazine in 0.03% DMSO final concentrations (a and b, respectively).

## Cocktails of oil, Simazine and DMSO

In the three experiments with nearly the same cocktails of oil, Simazine and DMSO, the difference between treatments increased with the time (Figure 6), but the extent of inhibition between specific treatments varied in each experiment (Figure 6a-6f, Tables 6,7,8).

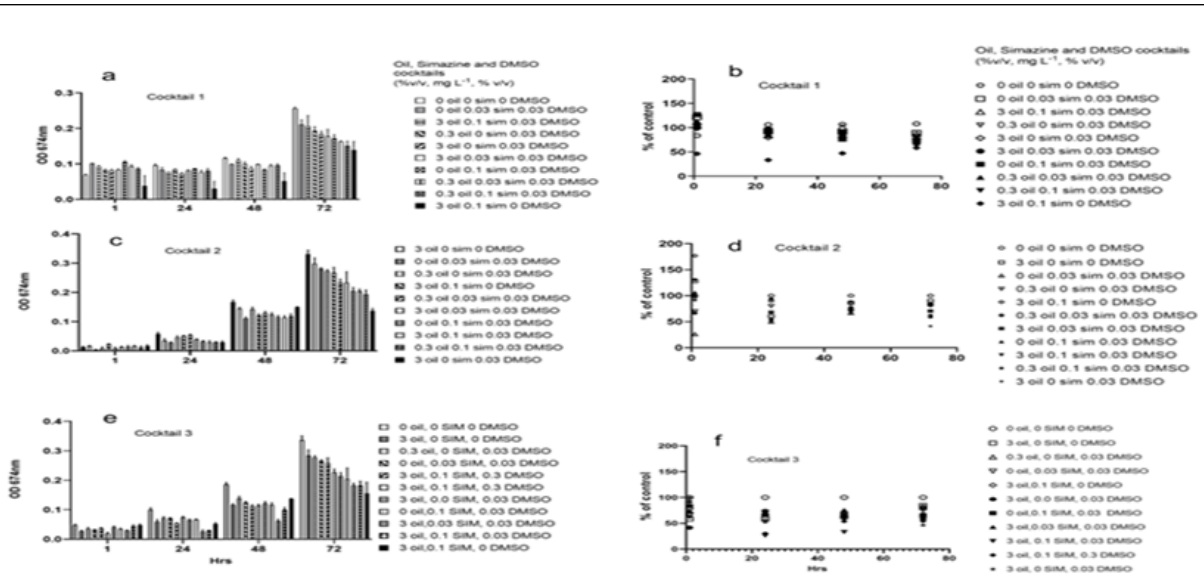


**Figure 6:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units and as percent of control (no pollutants), respectively, when grown in defined final concentrations cocktails of oil (% v/v), Simazine (mg $L^{-1}$) and DMSO (% v/v) final concentration (a and b, respectively) and OD 674 nm values (bars, left) and percentage of control (symbols, right); Experiments: 1(a,b), 2(c,d), 3(e,f).

Different pollutant cocktails inhibited the growth of the diatom, as reflected in the two growth models and the statistical analysis of the 72 h data. In the first cocktail experiment (Figure 6a and 6b), doubling time doubled and the logarithmic growth rate dropped to or below 50% relative to the control in the same three of nine combinations (Table 6).

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | Pollutant (Concentration) | Doubling time, estimated by the | Growth curve slope, based on the log- | Final sample average as % of control (RSE), | 2-W ANOVA results |

| | | exponential growth model (Equation 2) | linear growth model (equation 3) | t-test, Δ from control p-value, | |
|---|---|---|---|---|---|
| Units | | Hrs × generation$^{-1}$ | log OD hrs$^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |
| 1. | Cocktail 1 Oil Simazine (sim) DMSO | | | | Time p<0.0001 [70]; Treatment p=0.0008 [11]; Interaction p<0.0001 [7] |
| 1.1. | 0 oil 0 sim 0 DMSO | 31.83 | 0.009458/100 | 100/1.4 | |
| 1.2. | 0 oil 0.03 sim 0.03 DMSO | 46.13 | 0.00652/69 | 82/6.2, 0.024 * | |
| 1.3. | 3 oil 0.1 sim 0.03 DMSO | 43.83 | 0.006868/73 | 80/14.6, 0.197 | |
| 1.4. | 0.3 oil 0 sim 0.03 DMSO | 45.82 | 0.00657/69 | 76/5.7, 0.006 * | |
| 1.5. | 3 oil 0 sim 0.03 DMSO | 43.76 | 0.006879/73 | 72/4.7, 0.001 * | |
| 1.6. | 3 oil 0.03 sim 0.03 DMSO | 51.48 | 0.005848/62 | 69/10.8, 0.010 * | |
| 1.7. | 0 oil 0.1 sim 0.03 DMSO | 75.27 | 0.004/42 | 67/5.9, <0.001 * | |
| 1.8. | 0.3 oil 0.03 sim 0.03 DMSO | 64.55 | 0.004663/49 | 64/1.0, <0.001 * | |
| 1.9. | 0.3 oil 0.1 sim 0.03 DMSO | 71.43 | 0.004214/45 | 59/8.4, <0.001 * | |
| 1.10. | 3 oil 0.1 sim 0 DMSO | 23.29 | 0.01292/137 | 54/17.5, 0.009 * | |

**Table 6:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of cocktail experiment 1. Details in the legend to Table 3. *: significant at p<0.05.

Cell density in the final sampling of this trial (72 hrs) was significantly below the control in seven of the nine combinations, but in none of them, did final cell density fall below 50% of the control (Table 6). In the second cocktail experiment (Figure 6c and 6d, Table 7), in only five of ten combinations the 72 hrs cell density fell significantly below the control, and in the third cocktail experiment (Figure 6e and 6f, Table 8), in eight of ten combinations the 72 hrs cell density fell significantly below the control. In only two experiments, did one cocktail in each reduce growth<50% of control (2$^{nd}$ and 3$^{rd}$ cocktail experiments).

| | **A** | **B** | **C** | **D** | **E** |
|---|---|---|---|---|---|
| | **Pollutant (Concentration)** | **Doubling time, estimated by the exponential growth model (Equation 2)** | **Growth curve slope, based on the log-linear growth model (equation 3)** | **Final sample average as % of control (RSE), t-test, Δ from control p-value,** | **2-W ANOVA results** |
| Units | | Hrs × generation$^{-1}$ | log OD hrs$^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |
| 1. | **Cocktail 2 Oil Simazine (sim) DMSO** | | | | Time p<0.0001 [86]; Treatment p<0.0001 [3.4]; Interaction p<0.0001 [5.8] |
| 1.1. | 0 oil 0 sim 0 DMSO | 20.54 | 0.01466/100 | 100 (4.3) | |
| 1.2. | 3 oil 0 sim 0 DMSO | 19.04 | 0.01581/108 | 90 (6.4), 0.404 | |
| 1.3. | 0 oil 0.03 sim 0.03 DMSO | 16.27 | 0.0185/126 | 85 (1.5), 0.065 | |
| 1.4. | 0.3 oil 0 sim 0.03 DMSO | 20.63 | 0.01459/99 | 83 (2.1), 0.080 | |
| 1.5. | 3 oil 0.1 sim 0 DMSO | 20.56 | 0.01464/100 | 81 (6.6), 0.109 | |
| 1.6. | 0.3 oil 0.03 sim 0.03 DMSO | 22.74 | 0.01324/90 | 71 (4.8), 0.016 * | |
| 1.7. | 3 oil 0.03 sim 0.03 DMSO | 21.16 | 0.01423/97 | 71 (16.1), 0.118 | |

| | | | | | |
|---|---|---|---|---|---|
| 1.8. | 0 oil 0.1 sim 0.03 DMSO | 21.79 | 0.01382/94 | 62 (5.7), 0.005 * | |
| 1.9. | 3 oil 0.1 sim 0.03 DMSO | 21.65 | 0.0139/95 | 62 (3.7), 0.003* | |
| 1.10. | 0.3 oil 0.1 sim 0.03 DMSO | 22.78 | 0.01322/90 | 59 (7.5), 0.005 * | |
| 1.11. | 3 oil 0 sim 0.03 0.03 DMSO | 32.02 | 0.0094/64.1 | 40 (10), <0.001 * | |

**Table 7:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of cocktail experiment 2. Details in the legend to Table 3. *: significant at $p<0.05$.

| | **A** | **B** | **C** | **D** | **E** |
|---|---|---|---|---|---|
| | **Pollutant (Concentration)** | **Doubling time, estimated by the exponential growth model (Equation 2)** | **Growth curve slope, based on the log-linear growth model (equation 3)** | **Final sample average as % of control (RSE), t-test, Δ from control p-value,** | **2-W ANOVA results** |
| Units | | Hrs × generation$^{-1}$ | log OD hrs$^{-1}$/% of control | % of control (RSE), p value, | In square parenthesis: % of the total variation |
| 1. | **Cocktail 3 Oil Simazine (sim) DMSO** | | | | Time p<0.0001 [80]; Treatment p<0.0001 [8]; Interaction p<0.0001 [6.5] |
| 1.1. | 0 oil, 0 SIM 0 DMSO | 26.94 | 0.01117/100 | 100 (4.2) | |
| 1.2. | 3 oil, 0 SIM, 0 DMSO | 20.13 | 0.01495/134 | 84 (6.8), 0.074 | |
| 1.3. | 0.3 oil, 0 SIM, 0.03 DMSO | 24.13 | 0.01247/112 | 83 (2.0), 0.057 | |
| 1.4. | 0 oil, 0.03 SIM, 0.03 DMSO | 23.56 | 0.01278/114 | 79 (1.5), 0.0267 * | |
| 1.5. | 3 oil, 0.1 SIM, 0 DMSO | 26.94 | 0.01392/125 | 77 (6.8), 0.001 * | |
| 1.6. | 0.3 oil, 0.03 SIM, 0.03 DMSO | 25.26 | 0.01192/107 | 67 (4.9), 0.007 * | |
| 1.7. | 0 oil, 0.1 SIM, 0.03 DMSO | 28.78 | 0.01046/94 | 64 (5.5), 0.0044 * | |
| 1.8. | 3 oil,0.03 SIM, 0.03 DMSO | 29.03 | 0.01037/93 | 61 (18.4), 0.0275 * | |
| 1.9. | 3 oil, 0.1 SIM, 0.03 DMSO | 17.4 | 0.0173/155 | 54 (4.1), 0.001 * | |
| 1.10. | 3 oil, 0.1 SIM, 0 DMSO | 25.38 | 0.01186/106 | 54 (7.9), 0.035 * | |
| 1.11. | 3 oil, 0 SIM, 0.03 DMSO | 40.41 | 0.00745/67 | 46 (24.4), 0.0079 * | |

**Table 8:** Impact of the treatment on the doubling time (from the exponential growth model in Equation 2), logarithmic growth rate (from the semi-logarithmic equation 3) and the cell density on the last sampling of cocktail experiment 3. Details in the legend to Table 3. *: significant at $p<0.05$.

The 72 h data from the three experiments were combined, to get an impression of the synergy between the three chemicals (Figure 7). The control (no pollutants) provided the highest 72 hrs cell density in all three cocktail experiments. Oil inhibited the diatom 72 hrs cell densities only in the presence of Simazine and/or DMSO. In the latter case, the impact depended on the oil doze. Interestingly, the highest levels of Simazine and oil inhibited the diatoms regardless of the presence of the solvent, while at the intermediate Simazine level; oil increased the toxicity (Figure 7).

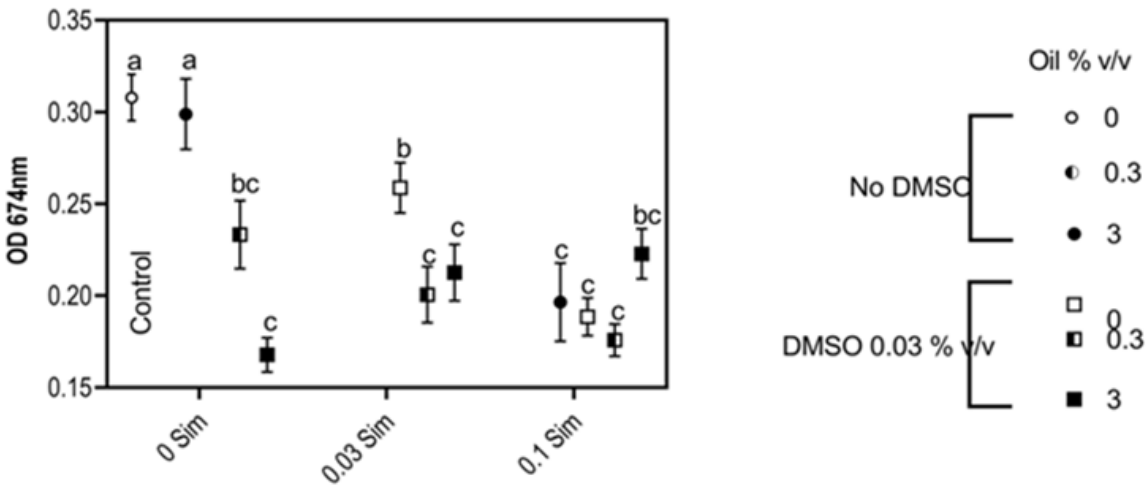


**Figure 7:** Algal (*Phaeodactylum tricornutum*) density in optical density (674 nm) units after 72 hrs of growth in media with defined concentrations of oil (% v/v), Simazine (mg $L^{-1}$) and DMSO (% v/v). Data from three cocktail experiments (not all combinations were tested).

## Discussion

Following the identification of the importance of the evaluation of mixtures' impacts in algal biotests with selected pesticidal compounds [20], the procedure proposed by Ma and Chen [21] to assess the toxicity was deemed impractical, due to the data variability, typical of such studies [9,11]. Nevertheless, the data in the present study provide insight into the toxicity of cocktails of pollutants to diatoms. The test diatom *Phaeodactylum tricornutum* was not sensitive to the examined pollutants in the ranges that were ecologically relevant to our data from the Black Sea. For instance, the algal growth rate was reduced to ≤ 50% of the control value at ≥ 0.1 mg $L^{-1}$, (i.e., about 0.5 µmole $L^{-1}$) of either herbicide. This value is too high to allow a practical application of the bio-test for concentrations of the herbicides found in our and others' [22] data from Black Sea coastal waters and other regions [23], which were usually lower by about two orders of magnitude.

The standard test used here can be compared with other tests. A sensitive approach depended on benthic diatoms from the studied ecosystem, whose health classification was estimated microscopically after 48 hrs exposures to the pollutants [24]. While the variability between replicates there was similar to ours, Atrazine inhibited some of the diatoms at concentrations as low as 0.05 mg $L^{-1}$. Weiner et al. tested the toxicity of Atrazine to several diatom species using microscope cell counts and determined 96 h EC50 values of about 0.045 to 0.091 mg $L^{-1}$. Other studies used the concentration of chlorophyll-*a* [6,7]. They found that, at a different level of DMSO (0.5 to 1% v/v), Atrazine began to be toxic at levels ≥ 0.03 mg $L^{-1}$. Like in some of our experiments, under some conditions, inexplicably the algae performed better under the toxins than in the control [7].Our approach, of measuring algal growth by optical density, was used in the testing of Atrazine toxicity to green microalgae, with similar results, with IC50 above 0.043 mg $L^{-1}$ and DMSO as a least toxic solvent [25].

The present tests are about as sensitive to the herbicides examined as in other published studies, such as a similar test with the herbicide DCMU, with a detection limit of ≥ 0.05 mg $L^{-1}$ (0.2 µmole $L^{-1}$) [15]. Concerning oil, in the presence of DMSO, the level of toxicity detection was ≥ 0.032% (v/v). Recently, toxicity (EC50 at 24 h and 96 h) of different oil mixtures that included solvents to the diatom *Chaetoceros muelleri* as measured by flow cytometry of cell density was above 2.5 g $L^{-1}$ (ca 0.3% v/v) [5]. In our data, oil without solvent was not toxic even at 10% (v/v). The impression that the variability in the impact of oil with no solvent diminished with time may reflect the salting-out process of the oil from the emulsion [26], since with 0.03% DMSO the variability in the impact increased with time, and oil toxicity was significant at levels ≥ 0.032% (v/v) [5]. These levels are all far above the maximal level of total hydrocarbons found in our Black Sea field data (≤ 0.0011% v/v), and most other hydrocarbon samples in water by others from that region (up to 15 mg $L^{-1}$, or 0.0017% v/v; [15]). In [27], the concentration of total petroleum hydrocarbons in the waters of the Gulf of Mexico after a major oil spill averaged 202 and reached 11400 mg $kg^{-1}$ of seawater, which are 0.02 to 1.14% v/v [27]. The sensitivity of the diatom in our data falls in this range, suggesting that the method is sensitive enough in such polluted regions. Of course, this conclusion should be qualified by the fact that crude oil is composed of numerous organic compounds, each with its solubility and toxicity in seawater [28].

## Conclusion

Our synergy data suggest that at a high concentration of the herbicide, the oil helps dissolve the herbicide and increase its bioavailability and thereby its toxicity to the diatom when without DMSO. On the contrary, the data from the two Simazine levels and two oil levels suggest, and this interesting observation requires further testing, that in the presence of DMSO, high oil may reduce Simazine toxicity, perhaps by removing some of it from the water.

Lower levels of oil might have increased synergistically the toxicity of Simazine as in, e.g., [8,23], and the solvent increased the toxicity of the oil.

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## Compliance with Ethical Standards

**Conflict of interest:** The authors declare that they have no conflict of interest.

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