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Effect of 2,4,6-Trinitrotoluene and 2,4-Dinitrotoluene on the Growth Rate and Photosynthetic Capacity of the Cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann

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The synthetic explosive 2,4,6-trinitrotoluene (TNT) is released to the environment as a consequence of its utilization at ammunition plants and mines, as well as the deterioration or decommissioning of old arsenals. Because TNT is only slightly soluble in water, its disposal during manufacturing and testing requires large amounts of hot water, and as much as 2,000 m^3 of TNT-contaminated water may be generated per day by a single ammunition plant (Maleki 1994). The wastewaters are usually directed to waste lagoons and streams, from where they enter the ground waters and constitute a serious pollution hazard for aquatic ecosystems (Maleki 1994). TNT exerts a harmful effect on many different organisms, from bacteria to humans, and has been demonstrated to be mutagenic in several *in vitro* test systems (Maleki 1994). In natural environments, TNT toxicity may be reduced by phenomena such as photodegradation and metabolic breakdown by microbes (Mabey et al. 1983), but the reduction of its harmful effects *in toto* will depend on the toxicity of its degradation products and metabolites such as 2,4-dinitrotoluene (2,4-DNT) (Esteve-Núñez et al. 2001). Although transformation of nitroaromatics (such as TNT) to their by-products has been shown to result in a decrease in toxicity toward a green alga (Tadros et al. 2000), there is still scant information on the toxic effects of these compounds.

In freshwater ecosystems, a large fraction of primary production is provided by both microalgae and cyanobacteria, which also play an important role in the nutrient cycles of such systems. Thus, any adverse impact on these foundation communities is likely to have effects on higher trophic levels, affecting the health of the whole system. The harmful effects of TNT on unicellular freshwater algae have been demonstrated (Tadros et al. 2000; López-Rodas et al 2001; García-Villada et al. 2002), however, little is known about the toxic effects of TNT or its by-products on cyanobacteria, some of the most widespread organisms inhabiting freshwater ecosystems (Falkowski and Raven 1997). Their remarkable ubiquity and their sensitivity to the toxic effects of pollutants (Nyström et al. 1999; Campanella et al. 2000) make them an ideal indicator of the presence of environmental contaminants, and suggest their possible utilization in biosensors. The main objective of the present study was to investigate and compare the effect

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of TNT and 2,4-DNT on the growth and photosynthesis of the cyanobacterium *Microcystis aeruginosa* (Kützing) Lemmermann.

MATERIALS AND METHODS

Experiments were carried out by using a *M. aeruginosa* strain (Ma7D) from the Algal Culture Collection of the Veterinary Faculty, Complutense University, Madrid. This strain was collected from a pristine lagoon in Doñana National Park (SW Spain) in April 2001. Isolation procedures and culture methods were as described in Carrillo et al. (2003).

Cells were grown in 100 mL culture flasks with 20 mL of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany) at 25° C under a continuous irradiance of 50 μmol photons m^{-2} s^{-1}, over the waveband 400-700 nm, supplied by daylight fluorescent tubes. The cultures were maintained in balanced growth by serial transfers of an inoculum to fresh medium twice each month.

The effect of TNT and 2,4-DNT on the maximum growth rate (m) was studied by exposing experimental cultures to increasing doses of each compound. Experimental cultures were established in glass test tubes with 1.5×10^5 cells from a mid-log exponentially growing culture. Stock solutions of TNT and 2,4-DNT (provided by Fábrica Nacional de Armas, La Marañosa, Ministry of Defense, Spain) were prepared in distilled water, according to standard security protocols, and added to the experimental cultures to provide concentrations of 0.16, 0.30, 0.50, 0.90, 1.70, 3.10 and 5.60 mg L^{-1}. The final volume in each test tube was 5 mL. Three replications of each concentration for each chemical, and six unexposed controls were prepared. After 3 d, the cell density in each culture was determined by using a haemocytometer (double Neubauer, Fortuna W.G. Co, Wertheim, Germany). Maximum growth rate was derived from the equation $N_t = N_0 e^{mt}$ (Crow and Kimura 1970), where N_t and N_0 are the cell number at time t and 0, respectively, and t = 3 d; therefore, m was computed as $\mathrm{Log_e}(N_3/N_0)/3$.

A Clark-type liquid-phase oxygen electrode (YSI 5331; Yellow Springs Instrument Co., OH, USA) was used to measure light-saturated net photosynthetic (NPS_{max}) and dark respiration (DR) rates. Irradiance-saturated gross photosynthetic rate (GPS_{max}, an estimator of photosynthetic capacity) was calculated as the sum of NPS_{max} and DR. Culture samples (8 mL) were incubated in a temperature-controlled (24 ± 0.1 °C) 9 mL chamber, gently stirred by a magnetic stirrer. The NPS_{max} was obtained by illuminating the sample with a saturating irradiance of 700 μmol m^{-2} s^{-1} over the waveband 400-700 nm, by means of a slide projector. The values of DR were measured by covering the chamber with a black plastic sheet. Both NPS_{max} and DR were derived from the linear change of the oxygen level as a function of time (5-10 min).Three replications of photosynthetic light response curves were made (Figure 1). Once the photosynthetic capacity was determined as previously described, the dose-

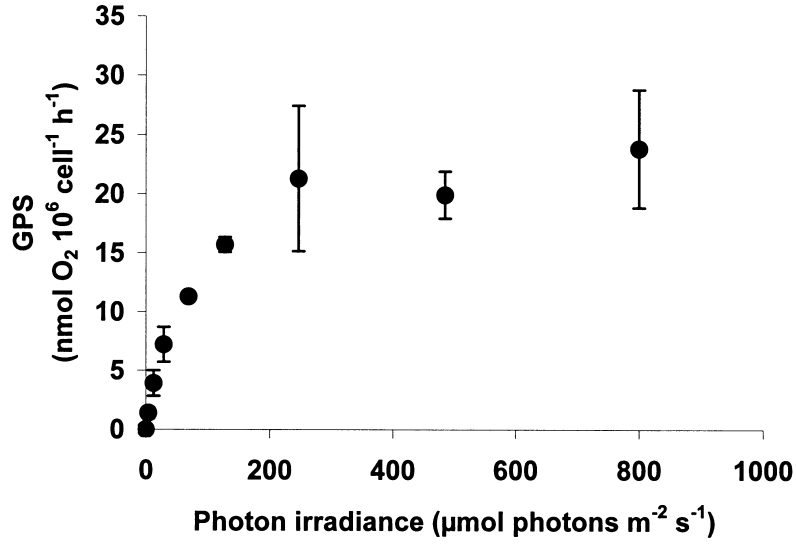

Figure 1. Gross photosynthetic rate (GPS) *versus* photon irradiance for *Microcystis aeruginosa* strain Ma7D (mean ± SD, $n = 3$).

effect curves for both chemicals were made. For this purpose, the same increasing doses of TNT and 2,4-DNT used for the previously described experiment were added to the chamber, and the GPS_{max} was obtained by illuminating the sample with a saturating irradiance of 700 µmol photons m^{-2} s^{-1}. For each chemical, three replications of each concentration and three unexposed controls were performed.

Data of *m* and GPS_{max} were converted to percentage of the respective controls. In order to test the effect of increasing concentrations of each chemical on *m* and GPS_{max}, the non-parametric statistical test of Mann-Whitney was performed. Additionally, the Wilcoxon signed-rank test was used for both measured parameters in order to evaluate differences between the effects of the two chemicals.

RESULTS AND DISCUSSION

The *m* of Ma7D strain exhibited little response to 2,4-DNT concentrations up to 1.70 mg L^{-1}; however, 3.10 mg L^{-1} and above completely inhibited *m* (Figure 2A). In the case of TNT, no significant effect was observed in cultures exposed to 0.16 and 0.30 mg L^{-1} (Figure 2A); higher concentrations significantly inhibited *m*, and cultures exposed to TNT concentrations of 1.70 mg L^{-1} and above showed no growth ($P>0.05$) (Figure 2A). These last results were in accordance with previous

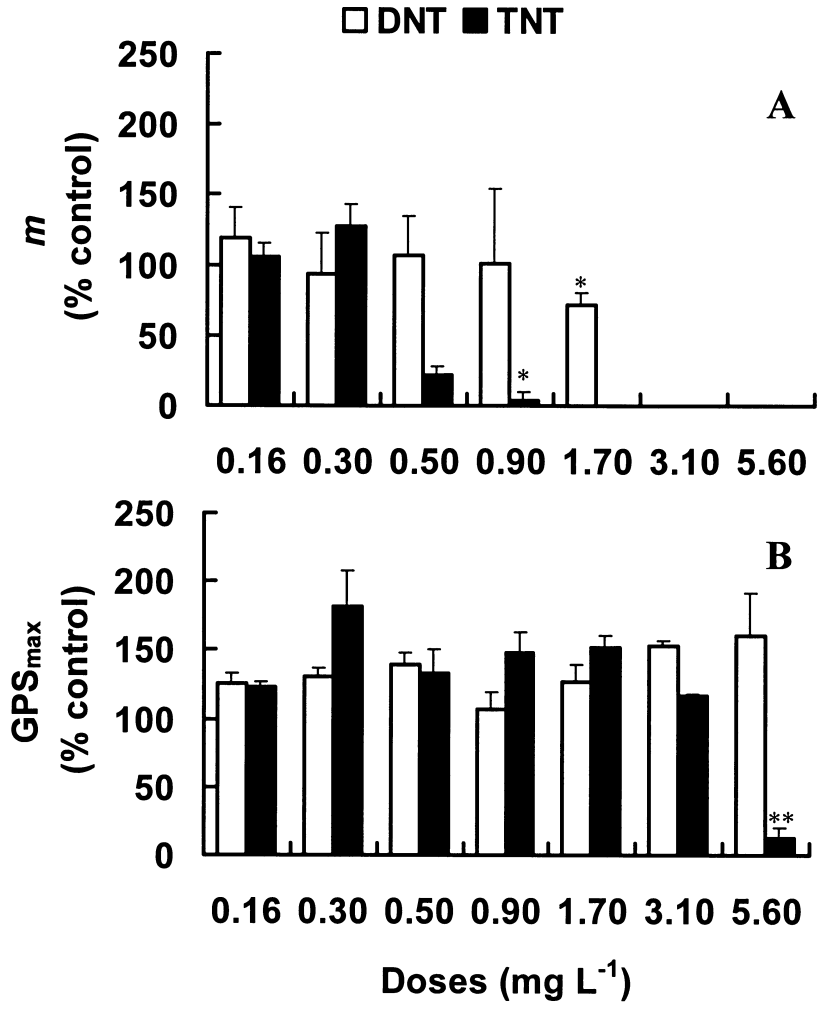

Figure 2. Dose effect of 2,4-DNT and TNT on (A) maximum growth rate (*m*) and (B) photosynthetic capacity (GPS_{max}) for *Microcystis aeruginosa* strain Ma7D (mean ± SD, $n = 3$). No detectable growth was found at 1.70 mg TNT L^{-1} and at 3.10 and 5.60 mg L^{-1} of both toxic compounds. * significant at $P>0.05$ and ** significant at $P>0.01$.

studies carried out with microalgae (Tadros et al. 2000; López-Rodas et al. 2001; García-Villada et al. 2002). Thus, the *m* of *M. aeruginosa* decreased with increasing doses of TNT, and even concentrations as low as 1.70 mg L^{-1} significantly inhibited growth. Levels of TNT close to 3.40 mg L^{-1} have been found in aquatic ecosystems (Talmage et al. 1999), so the severe decrease of *M. aeruginosa m* implies a challenge for the growth and survival of this species. On the other hand, it is well known that TNT can be denitrated and transformed into

2,4-DNT (Esteve-Núñez et al. 2001). Although Tadros et al. (2000) demonstrated a decrease in the toxic effects of nitroaromatic compounds in relation to the loss of their nitro groups, our results are difficult to reconcile with this assessment because no statistical significance was found between the effects of the two chemicals on *m* of *M. aeruginosa* in the range of concentrations tested here.

GPS_{max} was not significantly affected by 2 d of exposure to 2,4-DNT (Figure 2B) within the range of 2,4-DNTconcentrations used. A similar effect was observed for TNT, except the highest tested concentration (5.60 mg L^1) did affect the GPS_{max} ($P > 0.01$). As with *m*, no significant differences were found between TNT and 2,4-DNT in their effects on GPS_{max}, confirming the discrepancy between our data and those of Tadros et al. (2000).

According to these results, it seems that *m* was more sensitive to TNT and its by-products than the photosynthetic capacity, suggesting that TNT could also affect metabolic pathways other than those of photosynthesis. These results confirm those previously obtained by García-Villada et al. (2002) for the chlorophycean *Dictyosphaerium chlorelloides* (Naum.) Kom. and Perm., and they are in agreement with previous studies carried out with other microalgal species (Tadros et al. 2000). In particular, comparing our results with those of García-Villada (2002), we found that the growth of *M. aeruginosa* showed higher sensitivity to TNT than *D. chlorelloides*, as the *m* was significantly affected by concentrations of 0.90 and 1.70 mg L^{-1}, respectively. In contrast, GPS_{max} of *M. aeruginosa* remained unaffected at TNT concentrations that cause a severe decrease in GPS_{max} of *D. chlorelloides*, in which 50% GPS_{max}-inhibition was reached at only 2 mg L^{-1}.

Altamirano et al. (2004) suggested a new approach to detect TNT in water by analysing the chlorophyll fluorescence of photosystem II in *D. chlorelloides*. According to our results, *M. aeruginosa* could also be used as a biosensor in order to detect TNT-polluted waters by mean of the changes in *m* and photosynthetic capacity.

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