Differential sensitivity of aerobic gram-positive and gram-negative microorganisms to 2,4,6-trinitrotoluene (TNT) leads to dissimilar growth and TNT transformation: Results of soil and pure culture studies.

MARK E. FULLER AND JOHN F. MANNING, JR.

Environmental Research Division
Argonne National Laboratory
Argonne, IL 60439
USA

Corresponding author:

Mark E. Fuller
Environmental Research Division • ER/203-J070
Argonne National Laboratory
9700 South Cass Avenue
Argonne, IL 60439
Phone: (708) 252-1795. Fax: (708) 252-8895.
Electronic mail address: mark_fuller@qmgate.anl.gov

Running title: Differential sensitivity to TNT

Submitted: July 30, 1996

DISCLAIMER

ABSTRACT

The effects of 2,4,6-trintrootoluene (TNT) on indigenous soil populations and pure bacterial cultures were examined. The number of colony-forming units (CFU) appearing when TNT-contaminated soil was spread on 0.3% molasses plates decreased by 50% when the agar was amended with 67 μg TNT mL^{-1}, whereas a 99% reduction was observed when uncontaminated soil was plated. Furthermore, TNT-contaminated soil harbored a greater number of organisms able to grow on plates amended with greater than 10 μg TNT mL^{-1}. The percentage of gram-positive isolates was markedly less in TNT-contaminated soil (7%; 2 of 30) than in uncontaminated soil (61%; 20 of 33). *Pseudomonas aeruginosa, Pseudomonas corrugata, Pseudomonas fluorescens* and *Alcaligenes xylosoxidans* made up the majority of the gram-negative isolates from TNT-contaminated soil. Gram-positive isolates from both soils demonstrated marked growth inhibition when greater than 8-16 μg TNT mL^{-1} was present in the culture media. Most pure cultures of known aerobic gram-negative organisms readily degraded TNT and evidenced net consumption of reduced metabolites. However, pure cultures of aerobic gram-positive bacteria were sensitive to relatively low concentrations of TNT as indicated by the 50% reduction in growth and TNT transformation which was observed at approximately 10 μg TNT mL^{-1}. Most non-sporeforming gram-positive organisms incubated in molasses media amended with 80 μg TNT mL^{-1} or greater became unculturable, whereas all strains tested remained culturable when incubated in mineral media amended with 98 μg TNT mL^{-1}, indicating that TNT sensitivity is likely linked to cell growth. These results indicate that gram-negative organisms are most likely responsible for any TNT transformation in contaminated soil, due to their relative insensitivity to high TNT concentrations and their ability to transform TNT. Aerobic gram-positive organisms, however, are most likely

adversely affected by TNT contamination of soil and probably exist predominantly as resistant resting stages.

INTRODUCTION

The biodegradation of munitions has received increased attention recently, especially in light of the extensive contamination present at many military installations. For over 50 years, the biological transformation and degradation of 2,4,6-trinitrotoluene (TNT) and other munitions have been known to occur (27), although the observation was also made that high levels of TNT had the potential of inhibiting biological activity (28). Relatively recently, investigators have attempted to study aerobic TNT transformation in depth by using complex systems (6, 19) and pure cultures of *Pseudomonas* (11, 32), *Corynebacterium* (13), *Mycobacterium* (30), *Streptomyces* (24), and the white-rot fungus *Phanerochaete chrysosporium* (21, 29). Anaerobic biodegradation has been examined using consortia (5, 12), *Clostridium* (8, 20), *Desulfovibrio* (1, 3), *Veillonella* (20), an unidentified sulfidogenic bacterium (26) and the archaeobacterium *Methanococcus* (2). Klausmeier et al. (15) reported the effects of TNT on different classes of microbes (unidentified gram-positive sporeformers, actinomycetes and several genera of yeasts and fungi) and concluded that observable effects occurred at TNT concentrations greater than 50 $\mu g\ mL^{-1}$. Interestingly, no comprehensive results have been published indicating the TNT transformation capacity of specific microbial genera common to the soil environment or the potential effects of TNT on these genera. Furthermore, most examinations of TNT-contaminated soils have dealt with the isolation of specific pure or mixed cultures able to degrade TNT, rather than the *in situ* microbial community before, during, and after remedial efforts to biodegrade TNT. This research was undertaken to test the hypotheses that (i) aerobic gram-positive organisms are more susceptible to inhibition of both growth and TNT transformation capacity than aerobic gram-negative organisms, and; (ii) the microbial community of TNT-contaminated soil is dominated by

gram-negative organisms.

MATERIALS AND METHODS

Chemicals and media. All chemicals were reagent grade or purer. 2,4,6-Trinitrotoluene (TNT) was obtained from Chem Services, Inc. (West Chester, PA). 2-amino-4,6-dinitrotoluene (2ADNT) and 4-amino-2,6-dinitrotoluene (4ADNT) were supplied by the U. S. Naval Surface Warfare Center, Indian Head Division (Indian Head, MD). Mineral medium (MM) (50 mM; pH 6.8) consisted of the following (g L^{-1}): KH_2PO_4, 0.347; K_2HPO_4, 0.427; $(NH_4)_2SO_4$, 1.234; $MgSO_4 \cdot H_2O$, 0.460; $CaCl_2 \cdot 2H_2O$, 0.176; $FeSO_4 \cdot 7H_2O$, 0.001; and 5 mL of a solution of trace metals (mg L^{-1}: H_3BO_3, 60; $CoCl \cdot 6H_2O$, 40; $ZnSO_4 \cdot 7H_2O$, 20; $MnCl_2 \cdot 4H_2O$, 6; $NaMoO_4 \cdot 2H_2O$, 6; $NiCl_2 \cdot 6H_2O$, 4; $CuCl_2 \cdot 2H_2O$, 2) made in pure water (JT Baker, McGraw Park, IL). Soil extraction buffer (SEB) (50 mM; pH 6.8) consisted of 0.694 g of KH_2PO_4 and 0.854 g of K_2HPO_4 per liter of pure water. The standard culture medium was 0.3% molasses (v:v) in deionized water; agar was added at 20 g L^{-1} when needed. Media containing TNT were autoclaved, mixed for 24 hours to dissolve crystalline TNT, dispensed into appropriate containers and re-autoclaved.

Soil. Soil contaminated with TNT was excavated from the ridge-and-furrow area of Group 61 at the Joliet Army Ammunition Plant (JAAP). This area received large amounts of TNT-laden process water over a fifty year period, leaving the soil contaminated with residual TNT to a depth of 6-8 inches. Uncontaminated soil was taken from the upper bank of the ridge-and-furrow area. The initial TNT concentration in the contaminated soil was approximately 7800 $\mu g\ g^{-1}$, while the uncontaminated soil contained no detectable munitions compounds. Soil was air-dried, screened to remove roots and small rocks, rewetted to approximately 12%

moisture content (w:w), and stored at room temperature for 1 week prior to use.

Soil experiments. Soil (1.0 g dry weight) was added to 9 mL of SEB in a screw cap tube and shaken horizontally for 5 minutes. One milliliter of this soil suspension was serially diluted in SEB, and spread plates were made on half-strength tryptic soy agar and molasses agar amended with nominal concentrations of 0, 10, 25 and 67 μg TNT mL^{-1}. The remaining suspension was heat-shocked by placing it in an 80°C water bath for 15 minutes, followed by immersion in cold water. This suspension was also diluted and plated onto the same media. Plates were incubated at room temperature, and colonies were counted each day until the number of colony-forming units (CFU) stabilized. Representative colonies from all plates exhibiting growth were picked for purification and further testing.

After purification, isolates were gram stained. Isolates that produced anomalous results were stained again upon subculturing and the Ryu KOH test was also performed (25). Gram-negative isolates were identified by using the Biolog Identification System (Biolog, Inc., Hayward, CA) running MicroLog 1 (v. 3.5). All isolates were plated onto agar media amended with different concentrations of TNT to assess the sensitivity of the indigenous soil populations of gram-positive and gram-negative bacteria.

Pure culture experiments. The bacterial genera, source, and growth media are listed in Table 1. Organisms purchased from the American Type Culture Collection (ATCC) were screened for growth in molasses media; those organisms not growing were cultured in the recommended media. Bacteria were subcultured at least three times before being used in experiments, and repeated purity checks were conducted to ensure that anexic cultures were being tested. Several trials were conducted because of the large number of cultures screened.

In each trial, bacteria were inoculated into triplicate 30 mL screw cap tubes containing 10 mL of the appropriate medium containing 8 different concentrations

of TNT. Tubes were shaken at a 45° angle on a specially equipped orbital shaker (200 rpm; room temperature). Initial concentrations of TNT in each medium are listed in Table 4. The optical density (600 nm) of the cultures was monitored on a regular basis to assess growth. After two weeks of incubation, tubes were sampled for residual TNT and metabolite concentrations. One milliliter of the well mixed culture was spun for 2 minutes at 12,400 rpm in a microcentrifuge to pellet the cells. A 0.5 mL volume of the supernatant was added to 0.5 mL of acetonitrile, mixed by inverting and was analyzed as described below. Cultures exhibiting growth inhibition were analyzed for culturable cells by dropping 20 µL of the liquid from each of the three replicate tubes onto TNT-free molasses agar plates and examining the plates for growth for up to two weeks. Confirmatory trials were conducted with bacteria showing extensive TNT transformation of high initial TNT concentrations, as well as those gram-negative bacteria exhibiting TNT sensitivity.

Gram-positive bacteria were inoculated into molasses medium and MM amended with 0 and 80-98 µg TNT mL^{-1} to assess whether the apparent loss of culturability caused by elevated TNT concentrations was dependent upon the presence of utilizable carbon. The sensitivity to 2ADNT and 4ADNT, both at concentrations of 5 µg mL^{-1}, was also assessed in molasses medium and MM. After incubation for 2 weeks, culturability was assessed by dropping 10 µL aliquots onto TNT- and monoaminodinitrotoluene-free plates and examining them for colony formation for up to 2 weeks.

Analytical methods. TNT was analyzed by high performance liquid chromatography with a Water Associates (Milford, MA) liquid chromatograph equipped with two model 6000A solvent pumps, a model 990 variable-UV-array, multiple-wavelength detector set at 254 nm, a data module, and a model 600E system controller. The mobile phase was methanol:water (50:50). Sample aliquots (50 µL) were injected onto a Supelco LC-18 5 µm column (Bellefonte, PA) at room

temperature. The flow rate was 1.5 mL min^{-1}. Both quantitative and qualitative standards of TNT and metabolites were run periodically to validate retention time and conversion factors for each of the compounds.

Data analysis. Data were analyzed by ANOVA by using StatView (v. 4.0; Abacus Concepts Inc., Berkeley, CA). Treatment means were tested for significant differences at the 1% level by using the Fisher's protected least significant difference post hoc procedure. The Levenberg-Marquardt algorithm was used to fit logistic curves to growth and TNT transformation data from gram-positive organisms, from which effective concentrations causing 50% reduction in growth and TNT transformation ($EC_{50/growth}$ and $EC_{50/trans}$, respectively) were determined.

RESULTS

Soil experiments. The number of culturable heterotrophs growing on half-strength tryptic soy agar (TSA) and molasses agar was significantly greater ($P \leq 0.0001$) for uncontaminated soil than for TNT-contaminated soil (Figure 1A). Uncontaminated and TNT-contaminated soil harbored similar numbers of organisms able to grow on molasses agar amended with 10 μg TNT mL^{-1}, whereas TNT-contaminated soil contained 2 and 10 times more bacteria that were able to grow on molasses agar amended with 25 and 67 μg TNT mL^{-1}, respectively, than uncontaminated soil.

When soil was heat-shocked before plating, the number of bacteria from uncontaminated soil that grew on half-strength TSA were too numerous to count (> 3×10^6 CFU g^{-1}), compared with 2×10^4 CFU g^{-1} from TNT-contaminated soil. The number of culturable heterotrophs from uncontaminated soil growing on molasses agar and molasses agar amended with 10 μg TNT mL^{-1} were

significantly greater ($P \leq 0.0001$) than those from TNT-contaminated soil (Figure 1B). Bacteria able to grow on molasses agar amended with 25 and 67 μg TNT mL^{-1} were below detection (< 100 CFU g^{-1}) for uncontaminated soil, while TNT-contaminated soil contained approximately 1500 and 50 CFU g^{-1}, respectively, that were able to grow at these TNT concentrations.

After isolates from uncontaminated and TNT-contaminated soil were purified and successfully gram stained, it was readily apparent that uncontaminated soil was dominated by gram-positive bacteria (61% of the total), whereas the culturable heterotrophs in TNT-contaminated soil were overwhelmingly gram-negative (93% of the total) (Table 2). When soil was heat-shocked before plating, the percentage of gram-positives was high for both soils (100% and 81% of the total isolates, respectively). No gram-positive isolates were obtained from plates amended with 25 or 67 μg TNT mL^{-1} regardless of the source of inoculum. Approximately 10-12% of the isolates from both soils gave ambiguous results when gram stained. The identities of the gram-negative isolates from the uncontaminated and TNT-contaminated soils was different, as revealed by Biolog GN plates (Table 3), and a large proportion (≈ 50%) of the isolates from uncontaminated soil were not able to be identified. The growth of gram-positive soil isolates (n = 48) was completely inhibited on molasses plates amended with greater than 12.1 ± 4.5 μg TNT mL^{-1}, whereas 33 out of 34 of gram-negative isolates grew in the presence of the highest TNT concentration tested (67 μg mL^{-1}).

Pure culture experiments. All 31 strains tested were able to transform low initial TNT concentrations (Table 4). Net production of monoaminodinitrotoluenes was observed by 21 strains, while 10 strains evidenced net consumption of these compounds. The growth and TNT transformation capacity of gram-positive organisms were largely inhibited by initial TNT concentrations of approximately 10 μg mL^{-1}. Most gram-negative organisms grew well and degraded a substantial

percentage of the TNT even in the presence of initial TNT concentrations of 66 μg mL^{-1}. However, *Acinetobacter johnsonii*, *Cytophaga pectinovora* and *Flavobacterium odoratum* exhibited greatly reduced growth and TNT transformation capacity at higher initial TNT concentrations. In addition, *Alcaligenes eutrophus* grew at high TNT concentrations, but its transformation of TNT decreased steadily as the TNT concentration increased. Most notable among the gram-negative organisms were *Escherichia coli*, *Pseudomonas cepacia*, *Sphingomonas capsulata*, *Rahnella aquatilis* BFB and *Myxococcus xanthus*, which transformed virtually 100% of the TNT, regardless of the initial TNT concentration, and exhibited net consumption of 2ADNT and 4ADNT.

Gram-positive bacteria exhibited differential culturability when incubated in the presence of TNT (Table 5). Of the 14 gram-positive bacteria tested, 100% were culturable after 2 weeks of incubation in molasses media and MM, 12 of 14 (86%) remained culturable in MM plus 98 μg TNT mL^{-1} and only 3 of 14 (21%) were culturable from molasses media amended with 80 μg TNT mL^{-1} (Table 5). All 13 gram-positive organisms remained culturable after incubation in both molasses and MM amended with a combined concentration of 2ADNT and 4ADNT of 10 μg mL^{-1}. Of the gram-negative bacteria which failed to grow in the presence of high TNT concentrations, *Ac. johnsonii*, *Cy. pectinovora*, and *F. odoratum* cultures remained culturable after incubation with up to 66, 17 and 28 μg TNT mL^{-1}, respectively.

The data on percent maximum growth and percent initial TNT versus initial TNT concentration for individual gram-positive organisms were pooled and are presented in Figure 2. Using nonlinear regression on the pooled data revealed that the overall $EC_{50/growth}$ value was somewhat lower than the $EC_{50/trans}$ value (7.8 ± 0.4 vs. 9.5 ± 0.5 μg TNT mL^{-1}, respectively); this pattern was also observed when analyses were performed with data grouped by genera (Table 6). The regression

analysis of the data verified that the gram-positive bacteria tested were severely inhibited by TNT concentrations greater than 10 μg TNT mL^{-1}.

DISCUSSION

Both the pure culture and soil studies of this research substantiated the hypothesis that aerobic gram-positive bacteria are adversely affected by TNT. Aerobic gram-positive soil isolates were never observed to grow on molasses agar amended with confirmed concentrations of more than 20 μg TNT mL^{-1}, although the average concentration of TNT at which 100% inhibition was observed was 12.1 ± 4.5 μg TNT mL^{-1}. This observation roughly agrees with results from pure cultures of known aerobic gram-positive organisms in which 50% growth inhibition was seen at a level of TNT of 7.8 ± 0.4 μg TNT mL^{-1}. Of great interest was the fact that the $EC_{50/growth}$ and $EC_{50/trans}$ values of all the gram-positive genera tested fell within such a narrow range, indicating that the mechanism of inhibition is most likely due to the distinct characteristics of aerobic gram-positive bacteria as a group. Additionally, since organism culturability was more severely reduced by TNT in media containing readily utilizable carbon than in carbon-free media, the negative effects of TNT on aerobic gram-positive bacteria are probably linked to cell growth.

The pure culture component of this research greatly expands and refines the conclusions of previously published data concerning aerobic gram-positive organisms. Six pure cultures of *Bacillus* sp. isolated from soil were shown by Klausmeier et al. (15) to exhibit growth-inhibition in the presence of 50 μg TNT mL^{-1} or more, and TNT transformation was directly proportional to cell growth. However, the *B. cereus* and *B. subtilis* tested in this research neither grew nor transformed TNT when initial TNT concentrations were greater than 8 μg mL^{-1},

1 although the bacteria were both culturable after exposure to even 66 μg TNT mL^{-1}.
2 Pasti-Grigsby et al. (24) reported that several strains of actinomycetes isolated
3 from contaminated and uncontaminated soil, including *Streptomyces*
4 *chromofuscus*, grew in the presence of 75 or even 100 μg TNT mL^{-1}, depending
5 on the medium composition, and degraded 95% of the 25 μg TNT mL^{-1} when
6 grown in rich media. Klausmeier et al. (15) also reported severe growth inhibition
7 of actinomycetes in media amended with greater than 50 μg TNT mL^{-1}. The
8 present research, however, indicated that the growth of *S. albus* and *S. griseus* were
9 almost completely inhibited at TNT concentrations greater than 7 and 15 μg TNT
10 mL^{-1}, respectively, and that substantial transformation only occured below these
11 levels. The apparent contradiction between these results may be attributed to strain
12 differences, but since neither paper states that TNT concentrations in growth media
13 were confirmed, we would question whether the concentrations of TNT assumed to
14 be in the media of both Klausmeier et al. (15) and Pasti-Grigsby et al. (24) were
15 actually present. In fact, we have conducted quite extensive evaluations of the
16 effects of medium composition and preparation on final TNT concentrations, and
17 have found that TNT concentrations of 100 μg mL^{-1} are abiotically reduced to
18 below 10 μg mL^{-1} when added to rich media (brain heart infusion broth and tryptic
19 soy broth) and autoclaved (unpublished results). A brief mention of the inability of
20 a *Rhodococcus* sp. to transform 100 μg TNT mL^{-1} in a succinate-amended basal
21 media has been made by Dickel and Knackmuss (9). The four *Rhodococcus* spp.
22 tested in this research were inhibited with respect to TNT transformation at a
23 concentration of around 11 μg TNT mL^{-1}. Finally, to our knowledge this is the
24 first report of the growth and TNT transformation ability of the genera
25 *Arthrobacter*, *Corynebacterium* and *Micrococcus*.
26 Results obtained with TNT-contaminated and uncontaminated soil also indicate
27 that TNT is able to affect the microbial ecology of chronically contaminated

environments, in support of our second hypothesis. The shift from a gram-positive dominated to a gram-negative dominated community confirms the soil studies of Klausmeier et al. (15). However, this research does not agree with the older study, in that it clearly showed that the number of TNT-resistant bacteria in the TNT-contaminated soil was greater than that in uncontaminated soil and, further, that the number of heat-shock resistant organisms was significantly lower in TNT-contaminated soil compared with uncontaminated soil. The difference in results may be partially explained by noting that the older study employed experimentally contaminated soil (10,000 μg TNT g^{-1}; 25 day incubation), rather than soil with a five-decade history of heavy TNT contamination. The fact that gram-positive organisms are able to survive in soil contaminated with TNT concentrations far above the water solubility of TNT (≈ 100 μg mL^{-1}) is quite interesting, since the soil solution would most likely be saturated with TNT. Kaplan and Kaplan (14) observed previously that strains of *Bacillus* and actinomycetes are cultivable from soil containing concentrations of TNT as high as 25,000 μg mL^{-1} . Most likely, the ability of these organisms to form spores or other resistant stages is what allows them to survive. This ability is also believed to be the reason why the pure cultures of *B. cereus* and *B. subtilis* tested in this research were viable after exposure to high TNT concentrations.

Pure culture and soil experiments also substantiated the hypotheses that aerobic gram-negative organisms are, generally, more resistant to the inhibitory effects of TNT on growth and TNT transformation capacity. The published literature has reported TNT transformation by strains of *Alcaligenes* (7), *Desulfovibrio* (1, 3), *E. coli* (20), *Enterbacter* (7), *Pseudomonas* (4, 7, 11, 20, 23), and *Veillonella* (20), as well as the inhibitory effects of TNT on *Vibrio fisheri* (Microtox assay organism) (10). This research adds *Acinetobacter*, *Agrobacterium*, *Cytophaga*, *Flavobacterium*, *Klebsiella*, *Myxococcus*, *Rahnella* and *Sphingomonas* to the

genera of gram-negative bacteria that have been examined, so that a general assessment of this group of organisms can be made. Most gram-negative cultures did exhibit some inhibition of TNT transformation capacity at the higher TNT concentrations, although growth was not affected and in most cases was actually stimulated. Measurable concentrations of monoaminodintrotoluenes were also produced and were not degraded further. Some strains, including *E. coli*, *R. aquatilis* BFB, *Ps. cepacia*, *Sp. capsulata* and *Mx. xanthus* performed very well, degrading all the added TNT and producing no detectable metabolites. Cultures of *A. eutrophus* evidenced no transformation above initial TNT concentrations of 7 μg mL^{-1}, while *Cy. pectinovora* and *Ac. johnsonii* were growth inhibited and did not transform TNT when initial concentrations were greater than 13 and 33 μg mL^{-1}, respectively. These results are in agreement with the research already cited, especially that of McCormick et al. (20), which reported wide variability between strains. Of the gram-negative bacteria isolated from TNT-contaminated and uncontaminated soil, all were able to grow on molasses agar amended with up to 67 μg TNT mL^{-1}. However, it was apparent that TNT-contaminated soil was dominated by a few pseudomonads -- *Ps. aeruginosa*, *Ps. corrugata*, and *Ps. fluorescens* (type B, F and G) -- and by *Al. xylosoxydans* (ss den/pie), while uncontaminated soil exhibited no dominance of one species over another.

Several explanations are possible for the differential sensitivity of aerobic gram-positive and gram-negative bacteria to TNT, and these explanations are currently under investigation in our laboratory. It cannot be generalized that gram-positive organisms are simply sensitive to nitro-substituted aromatic compounds, because several of these compounds (*p*-nitrophenol, 2,4-dinitrophenol, 2,4,6-trinitrophenol, 2,4-dinitrotoluene, trinitrobenzene, etc.) are transformed or completely mineralized at relatively high concentrations (> 100 μg mL^{-1}) by aerobic gram-positive bacteria (9, 13, 16, 17). Also, the anaerobic gram-positive

bacterium *Clostridium pastuerianum* (20) and an anaerobic coculture of gram-positive organisms (8) have been shown to grow and transform TNT at concentrations of 100 μg mL^{-1}. The most obvious reason for the difference between aerobic gram-positive and gram-negative bacteria is that the structure and composition of the cell wall and the presence of an outer membrane somehow protects gram-negative organisms from TNT. Gram-positive cell walls may simply be more permeable to TNT than the cell wall and outer membrane of gram-negative organisms, thus allowing more TNT to enter and disrupt proper cell functioning. The TNT may interfere with the proper assembly of the gram-positive cell wall specifically during growth because TNT appears to affect only actively growing cells. The possibility also exists that the resistant gram-negative bacteria may simply be less TNT-permeable than sensitive organisms, or they may possess either (a) active transport systems which readily move any TNT that gets into the cell back outside the cell or (b) enzyme which detoxify TNT. These three reasons seem more likely because TNT is structurally similar to the antimicrobial agent chloramphenicol, which is known to lose its effectiveness against strains with chloramphenicol-impermeable cell membranes (18, 31), chloramphenicol efflux pumps (18, 22), or enzymes that deactivate chloramphenicol intracellularly (31). Clearly this phenomenon needs to be examined in more depth. The existence of TNT-sensitive gram-negative organisms (*Ac. johnsonii, Cy. pectinovora* and *F. odoratum*) and TNT-insensitive anaerobic gram-positive organisms (*Cl. pastuerianum*) will assist in these efforts.

Considering that most TNT-contaminated soils have concentrations far in excess of the water solubility of TNT, we can reasonably assume that the soil solution is usually saturated with TNT and, hence, that indigenous aerobic gram-positive organisms are likely to be severely inhibited with respect to both their growth and contribution to TNT transformation.

ACKNOWLEDGMENTS

We wish to thank Michele Gurgas and Rip Warner, two Science and Engineering Research Semester students, for their excellent assistance in conducting these experiments.

This work was supported by the U.S. Department of Defense Army Environmental Center, through interagency agreement MIPR4330 with the U.S. Department of Energy. Argonne National Laboratory is operated by the University of Chicago for the U.S. Department of Energy under contract W-31-109-Eng-38.

REFERENCES

1. **Boopathy, R., and C. Kulpa**. 1992. Trinitrotoluene (TNT) as a sole nitrogen source for a sulfate-reducing bacterium *Desulfovibrio* sp. (B strain) isolated from an anaerobic digestor. Current Microbiol. **25:**235-241.

2. **Boopathy, R., and C. Kulpa**. 1994. Biotransformation of 2,4,6-trinitrotoluene (TNT) by a *Methanococcus* sp. (strain B) isolated from a lake sediment. Can. J. Microbiol. **40:**273-278.

3. **Boopathy, R., C. Kulpa, and M. Wilson**. 1993. Metabolism of 2,4,6-trinitrotoluene (TNT) by *Desulfovibrio* sp. (B strain). Appl. Microbiol. Biotechnol. **39:**270-275.

4. **Boopathy, R., J. Manning, Jr., C. Montemagno, and C. Kulpa**. 1994. Metabolism of 2,4,6-trinitrotoluene by a *Pseudomonas* consortium under aerobic conditions. Current Microbiol. **28:**131-137.

5. **Boopathy, R., M. Wilson, and C. Kulpa**. 1993. Anaerobic removal of 2,4,6-trinitrotoluene (TNT) under different electron accepting conditions: laboratory study. Water Environment Research **65:**271-275.

6. **Bradley, P., F. Chapelle, J. Landmeyer, and J. Schumacher**. 1994. Microbial transformation of nitroaromatics in surface soils and aquifer materials. Applied and Environmental Micrbiology **60:**2170-2175.

7. **Collie, S., K. Donnelly, B. Bae, R. Autenrieth, and J. Bonner**. 1995.

Degradation of 2,4,6-trinitrotoluene (TNT) in an aerobic reactor. Chemosphere **31**:3025-3032.

8. **Crawford, R.** 1995. Biodegradation of nitrated munition compounds and herbicides by obligately anaerobic bacteria. p. 87-98. *In* J. Spain (ed.), Biodegradation of nitroaromatic compounds, Plenum Press, New York.

9. **Dickel, O., and H. Knackmuss.** 1991. Catabolism of 1,3-dinitrobenzene by *Rhodococcus* sp. QT-1. Arch. Microbiol. **157**:76-79.

10. **Drzyzga, O., T. Gorontzy, A. Schmidt, and K. Blotevogel.** 1995. Toxicity of explosives and related compounds to the luminescent bacterium *Vibrio fischeri* NRRL-B-11177. Archives of Environmental Contamination and Toxicology **28**:229-235.

11. **Duque, E., A. Haidour, F. Godoy, and J. Ramos.** 1993. Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. **175**:2278-2283.

12. **Funk, S., D. Roberts, D. Crawford, and R. Crawford.** 1993. Initial-phase optimization for bioremediation of munition compound-contaminated soils. Appl. Environ. Microbiol. **59**:2171-2177.

13. **Gundersen, K., and H. Jensen.** 1956. A soil bacterium decomposing organic nitro-compounds. Acta Agricultura Scandia **6**:100-114.

14. **Kaplan, D., and A. Kaplan.** 1982. Thermophilic biotransformations of

2,4,6-trinitrotoluene under simulated composting conditions. Appl. Environ. Microbiol. **44:**757-760.

15. **Klausmeier, R., J. Osmon, and D. Walls.** 1974. The effect of trinitrotoluene on microorganisms. Dev. Ind. Microbiol. **15:**309-317.

16. **Lenke, H., and H. Knackmuss.** 1992. Initial hydrogenation during catabolism of picric acid by *Rhodococcus erythropolis* HL 24-2. Appl. Environ. Microbiol. **58:**2933-2937.

17. **Lenke, H., D. Pieper, C. Bruhn, and H. Knackmuss.** 1992. Degradation of 2,4-dinitrophenol by two *Rhodococcus* strains, HL 24-1 and HL 24-2. Appl. Environ. Microbiol. **58:**2928-2932.

18. **Li, X.** 1994. Role of efflux pump(s) in intrinstic resistance of *Pseudomonas aeruginosa* resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. **38:**1732-1741.

19. **Manning, J., Jr., R. Boopathy, and C. Kulpa.** July 1995. A laboratory study in support of the pilot demonstration of a biological soil slurry reactor. Argonne National Laboratory • Environmental Research Division. Report# SFIM-AEC-TS-CR-94038.

20. **McCormick, N., F. Feeherry, and H. Levinson.** 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. **31:**949-958.

21. **Michels, J., and G. Gottschalk.** 1994. Inhibition of the lignin peroxidase of *Phanerochaete chrysosporium* by hydroxylamino-dinitrotoluene, an early intermediate in the degradation of 2,4,6-trinitrotoluene. Appl. Environ. Microbiol. **60:**187-194.

22. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets - permeability barriers. Science **264:**382-388.

23. **Osmon, J., and R. Klausmeier.** 1972. The microbial degradation of explosives. Dev. Ind. Microbiol. **14:**247-252.

24. **Pasti-Grigsby, M., T. Lewis, D. Crawford, and R. Crawford.** 1996. Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from TNT-contaminated and uncontaminated environments. Appl. Environ. Microbiol. **62:**1120-1123.

25. **Powers, E.** 1995. Efficacy of the Ryu nonstaining KOH technique for rapidly determining gram reactions of food-borne and waterborne bacteria and yeasts. Appl. Environ. Microbiol. **61:**3756-3758.

26. **Preuß, A., J. Fimpel, and G. Diekert.** 1993. Anaerobic transformation of 2,4,6-trinitrotoluene (TNT). Arch. Microbiol. **159:**345-353.

27. **Ruchhoft, C., M. LeBosquet, Jr., and W. Meckler.** 1945. TNT wastes from shell-loading plants. Ind. Eng. Chem. **37:**937-943.

28. **Schott, S., C. Ruchhoft, and S. Megregian.** 1943. TNT wastes. Ind. Eng.

Chem. **35:**1122-1127.

29. **Spiker, J., D. Crawford, and R. Crawford.** 1992. Influence of 2,4,6-trinitrotoluene (TNT) concentration on the degradation of TNT in explosive-contaminated soils by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **58:**3199-3202.

30. **Vorbeck, C., H. Lenke, P. Fischer, and H. Knackmuss.** 1994. Identification of a hydride-Meisenheimer complex as a metabolite of 2,4,6-trinitrotoluene by a *Mycobacterium* strain. J. Bacteriol. **176:**932-934.

31. **Wolin, M., and T. Miller.** 1985. Physical agents, disinfectants, and chemotherapeutic agents. p. 129-160. *In* B. Freeman (ed.), Burrow's textbook of microbiology. 22nd Edition. W.B. Saunders Company, Philadelphia.

32. **Won, W., R. Heckly, D. Glover, and J. Hoffsommer.** 1974. Metabolic disposition of 2,4,6-trinitrotoluene. Appl. Microbiol. **27:**513-516.

Table 1. Bacterial cultures, sources and growth media used in this research.

Organism	Source	Medium
Gram-positive		
Arthrobacter globiformis	ATCC (8010)	0.3% molasses
Arthrobacter sp. RP17	CA delta soil	0.3% molasses
Bacillus cereus	ATCC (14579)	0.3% molasses
Bacillus subtilis	ATCC (6051)	0.3% molasses
Corynebacterium glutamicum	ATCC (13032)	0.3% molasses
Corynebacterium sp. Nap2	CA agricultural soil	0.3% molasses
Micrococcus luteus	ATCC (4698)	nutrient broth
Rhodococcus erythropolis	ATCC (4277)	0.3% molasses
Rhodococcus globerulus	ATCC (25714)	0.3% molasses
Rhodococcus rhodocrous	ATCC (13808)	0.3% molasses
Rhodococcus sp. TF2	CA agricultural soil	0.3% molasses
Streptomyces albus	ATCC (3004)	0.3% molasses
Streptomyces griseus	ATCC (23345)	0.3% molasses
SP1b (coryneform)	CA agricultural soil	0.3% molasses
Gram-negative		
Acinetobacter johnsonii	ATCC (17909)	0.3% molasses
Agrobacterium sp. 2PC	soil-slurry bioreactor	0.3% molasses
Alcaligenes eutrophus	ATCC (17697)	0.3% molasses
Cytophaga pectinovora	ATCC (19366)	0.3% molasses
Escherichia coli	ATCC (11775)	0.3% molasses
Flavobacterium odoratum	ATCC (4651)	nutrient broth
Klebsiella sp. 1PC	soil-slurry bioreactor	0.3% molasses
Myxococcus xanthus	ATCC (25565)	S P Medium (ATCC #432)†
Pseudomonas aeruginosa	ATCC (10145)	0.3% molasses
Pseudomonas cepacia	ATCC (25416)	0.3% molasses

Pseudomonas fluorescens	ATCC (13525)	0.3% molasses
Pseudomonas putida	ATCC (12633)	0.3% molasses
Pseudomonas sp. Tol1A	CA rangeland soil	0.3% molasses
Pseudomonas sp. JS150	J. Spain	0.3% molasses
Pseudomonas sp. DFC49	G. Sayler	0.3% molasses
Rahnella aquatilis BFB	IL stream sediment	0.3% molasses
Sphingomonas capsulata	ATCC (14666)	0.3% molasses

†Made without raffinose, but 2x galactose and sucrose.

Table 2. Number of gram-positive and gram-negative bacterial isolates obtained from uncontaminated and TNT-contaminated soil plated on different media†.

			Medium¥					
Pretreatment	Soil	Gram	TSA	Mol	Mol/10	Mol/25	Mol/67	Total
None	Uncontaminated	Positive	11	6	3	0	0	20
		Negative	2	1	2	4	4	13
	Contaminated	Positive	2	0	0	0	0	2
		Negative	6	6	6	4	6	28
Heat-shocked	Uncontaminated	Positive	4	2	5	0	0	11
		Negative	0	0	0	0	0	0
	Contaminated	Positive	7	4	6	0	0	17
		Negative	0	1	0	2	1	4

† Isolates yielding inconclusive gram stains are not included.

¥ Medium abbreviations: TSA = half-strength tryptic soy agar; Mol = 0.3% molasses; Mol/X = 0.3% molasses + X μg TNT mL^{-1}.

Table 3. Gram-negative bacterial isolates obtained from uncontaminated and TNT-contaminated soil. Number in parentheses indicate the number of isolates identified as the specified strain using Biolog GN plates.

Uncontaminated soil	TNT-contaminated soil
Agrobacterium rhizogenes A (1)	*Alcaligenes xylosoxydans* ss den/pie (5)
Pseudomonas fluorescens type B (2)	*Comamonas testosteroni* (1)
Pseudomonas tolaasii (1)	*Pseudomonas aeruginosa* (7)
Sphingomonas paucimobilis A (1)	*Pseudomonas corrugata* (5)
Xanthomonas maltophila (1)	*Pseudomonas fluorescens* type B (2)
	Pseudomonas fluorescens type F (3)
	Pseudomonas fluorescens type G (1)
	Pseudomonas nitroreducens (1)
	Pseudomonas stutzeri (1)
	Pseudomonas viridiflava B (1)
	Serpens flexibilis (1)

Table 4. Percent maxiumum growth and percent TNT transformed by gram-positive and gram-negative bacteria grown in the presence of different initial TNT concentrations. Net monoaminodinitrotoluene production (consumption) in cultures with the highest initial TNT cocnentration is given in the last column.

Organism	% maximum growth with initial TNT concentration of ($\mu g\ mL^{-1}$)†								% TNT transformed with initial TNT concentration of ($\mu g\ mL^{-1}$)							2ADNT and 4ADNT
Gram-positive	0	3	7	8	13	19	33	66	3	7	8	13	19	33	66	($\mu g\ mL^{-1}$)¥
A. globiformis	100	98	94	6	7	12	6	9	100	100	2	0	2	7	17	3
Arthrobacter sp. RP17[a]	100	98	94	73	1	2	2	1	100	100	100	2	1	0	0	19
B. cereus	100	78	74	0	6	34	2	7	10	100	0	0	4	8	19	3
B. subtilis	99	100	100	74	7	22	5	10	84	93	94	0	0	1	18	4
C. glutamicum	100	98	97	64	21	31	3	8	99	98	12	0	4	1	17	4
Corynebacterium sp. Nap2	100	88	80	38	49	12	4	7	100	100	100	100	15	29	0	(3)
M. luteus[b]	100	33	85	49	5	4	3	2	100	96	78	23	6	15	6	16
Rh. erythropolis	100	75	51	39	39	18	4	8	100	95	72	58	59	14	15	4
Rh. globerulus	100	59	61	0	9	32	5	8	92	88	62	3	0	15	16	4
Rh. rhodocrous	100	70	81	46	9	13	4	5	85	84	57	12	4	16	13	4
Rhodococcus sp. TF2	100	86	69	44	9	11	4	5	97	95	66	22	4	31	3	(2)
S. albus	100	65	64	2	7	34	4	16	94	89	59	19	9	6	9	5
S. griseus	100	61	72	39	29	30	4	5	100	100	97	94	68	1	9	5
SP1b (coryneform)[c]	100	85	47	38	42	5	5	4	100	94	71	68	22	9	10	3
Gram-negative																
Ac. johnsonii	58	57	54	50	57	65	100	7	100	100	100	100	100	100	20	3
Agrobacterium sp. 2PC[c]	98	100	96	95	89	72	78	69	100	100	100	98	98	100	100	11
Al. eutropuhus	100	89	92	84	88	86	76	73	95	80	14	32	37	26	27	2
Cy. pectinovora[c]	100	97	89	92	95	1	4	2	100	100	100	100	15	9	8	3

E. coli	41	49	38	33	37	39	46	100	100	100	100	100	100	100	100	(5)
F. odoratum[b]	100	99	86	86	85	69	7	4	100	100	100	100	100	63	0	26
Klebsiella sp. 1PC[c]	100	93	90	90	87	84	87	90	100	100	100	100	100	97	58	4
Mx. xanthus[d]	100	100	96	92	99	98	85	82	100	100	100	100	100	100	100	1
Ps. aeruginosa	77	83	81	79	94	93	98	100	99	97	78	87	88	80	52	1
Ps. cepacia	85	77	81	83	89	79	81	100	100	100	97	100	99	100	98	(7)
Ps. fluorescens	62	58	59	63	71	51	67	100	82	85	82	85	77	75	73	(3)
Ps. putida	81	83	82	79	87	85	78	100	66	68	71	71	67	58	65	(3)
Pseudomonas sp. Tol1A	96	97	100	92	99	97	92	94	76	75	57	65	56	56	46	1
Pseudomonas sp. JS150	100	92	90	71	84	86	77	77	100	100	99	99	95	90	65	(5)
Pseudomonas sp. DFC49	100	99	97	88	97	95	91	76	100	100	100	100	100	100	100	(2)
R. aquatilis BFB	100	97	99	83	92	91	91	94	100	100	100	99	100	99	100	(5)
Sp. capsulata[c]	100	91	83	80	78	72	64	63	100	100	100	100	100	100	100	(9)

† Percent maxium growth is the maximum growth observed at each TNT concentration divided by the maximum growth across all TNT concentrations multiplied by 100.

¥ Values represent the combined net production (consumption) of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene in media with the highest initial TNT concentrations. Numbers in parentheses represent net consumption of these compounds.

[a] Initial TNT ($\mu g\ mL^{-1}$): 0, <1, 1, 3, 5, 10, 21, 50.

[b] Initial TNT ($\mu g\ mL^{-1}$): 0, 2, 4, 6, 9, 15, 28, 60.

[c] Initial TNT ($\mu g\ mL^{-1}$): 0, 1, 4, 7, 10, 17, 35, 73.

[d] Initial TNT ($\mu g\ mL^{-1}$): 0, 4, 8, 13, 17, 23, 45, 64.

Table 5. The effetc of medium composition on the culturability of gram-positive bacteria after incubation with TNT and/or monoaminodinitrotoluenes.

	Culturable cells present after 2 weeks of incubation in†					
Organism	Mol	Mol/80	MM	MM/98	MM/AM	Mol/AM
A. globiformis	+	-	+	+	+	+
Arthrobacter sp. RP17	+	-	+	+	+	+
B. cereus	+	-	+	+	+	+
B. subtilis	+	+	+	+	+	+
C. glutamicum	+	-	+	+	+	+
Corynebacterium sp. Nap2	+	-	+	+	+	+
M. luteus[a]	+	+	+	-	ND[b]	ND
Rh. erythropolis	+	-	+	+	+	+
Rh. globerulus	+	-	+	+	+	+
Rh. rhodocrous	+	+	+	+	+	+
Rhodococcus sp. TF2	+	-	+	+	+	+
S. albus	+	-	+	-	+	+
S. griseus	+	-	+	+	+	+
SP1b (coryneform)	+	-	+	+	+	+

† Medium abbreviations: Mol = 0.3% molasses; Mol/80 = 0.3% molasses + 80 μg TNT mL^{-1}; MM = mineral medium with no added carbon; MM/98 = mineral medium + 98 μg TNT mL^{-1}; MM/AM = mineral medium + 5 μg 2ADNT mL^{-1} and 5 μg 4ADNT mL^{-1}; Mol/AM = 0.3% molasses + 5 μg 2ADNT mL^{-1} and 5 μg 4ADNT mL^{-1}.

[a] *M. luteus* was incubated in nutrient broth.

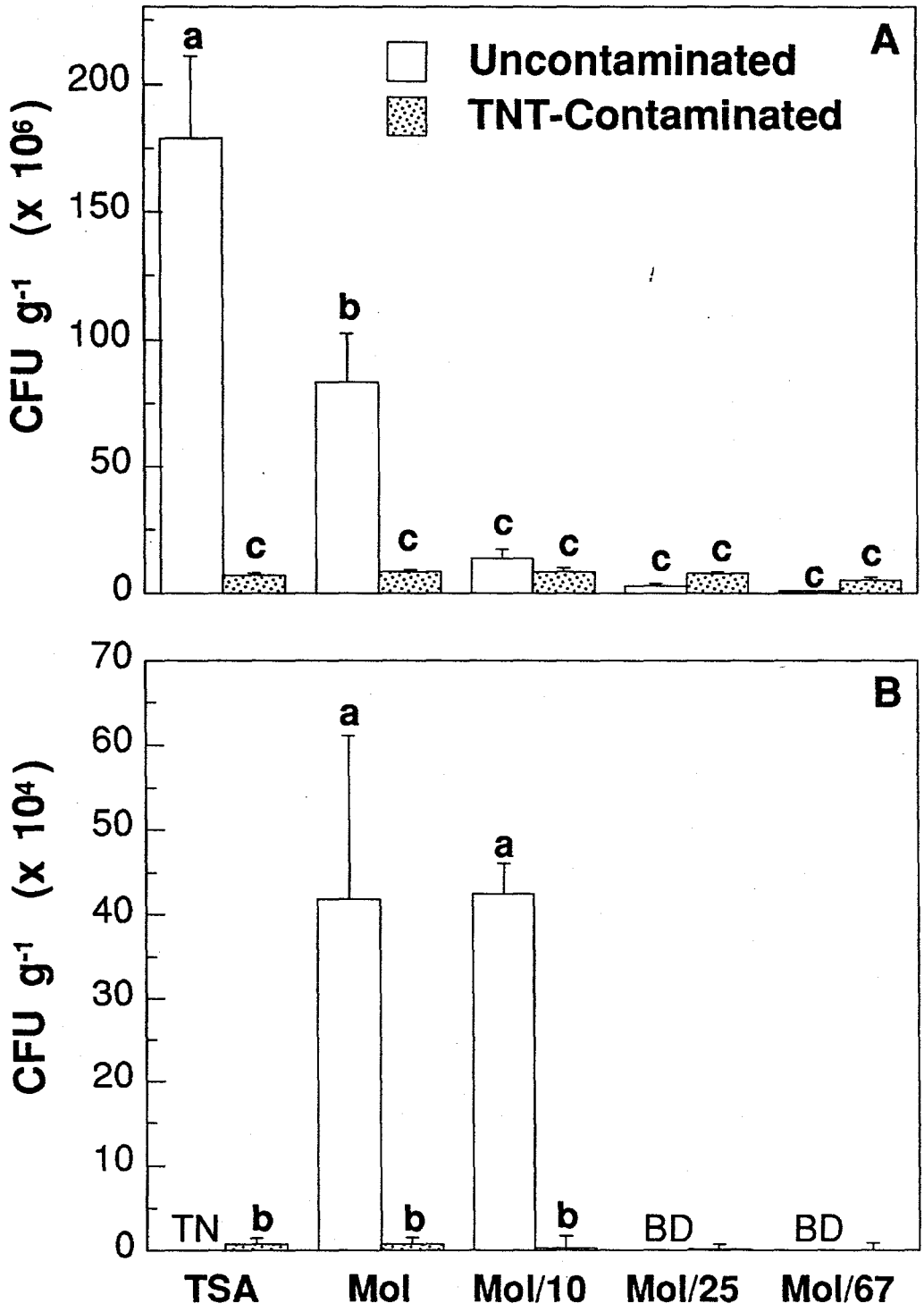
[b] ND, not determined.

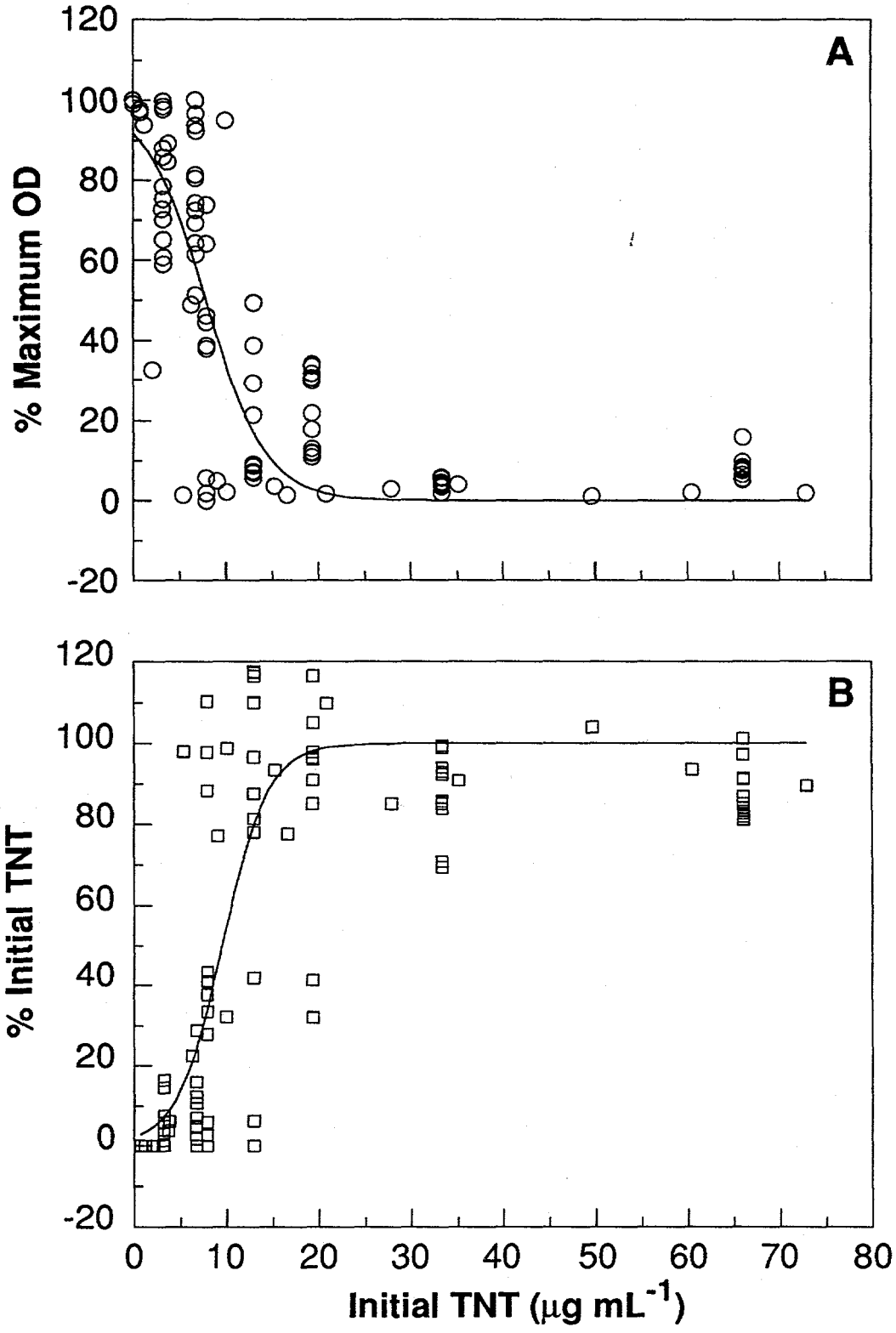
Table 6. Calculated $EC_{50/growth}$ and $EC_{50/trans}$ for pooled data from gram-positive organisms and for specific gram-positive genera as determined by fitting logistic curves to pooled data using nonlinear regression analysis. Goodness of fit values are given in parentheses.

Genus	Isolates Tested	$EC_{50/growth}$ ($\mu g\ mL^{-1}$)		$EC_{50/trans}$ ($\mu g\ mL^{-1}$)	
Arthrobacter	2	5.6 ± 0.9	($R^2 = 0.77$)	6.0 ± 1.0	($R^2 = 0.73$)
Bacillus	2	7.6 ± 0.3	($R^2 = 0.79$)	7.8 ± 0.3	($R^2 = 0.76$)
Corynebacterium	2	11.0 ± 1.0	($R^2 = 0.88$)	11.6 ± 2.3	($R^2 = 0.56$)
Micrococcus	1	6.0 ± 0.1	($R^2 = 0.99$)	7.6 ± 0.4	($R^2 = 0.97$)
Rhodococcus	4	7.3 ± 0.6	($R^2 = 0.84$)	10.3 ± 0.7	($R^2 = 0.78$)
Streptomyces	2	6.9 ± 1.2	($R^2 = 0.70$)	15.9 ± 2.3	($R^2 = 0.74$)
Pooled data	14	7.8 ± 0.4	($R^2 = 0.74$)	9.5 ± 0.5	($R^2 = 0.64$)

Figure 1. Culturable heterotrophs in uncontaminated (□) and TNT-contaminated soil (▩). See Materials and Methods for media descriptions. A, total heterotrophs; B, heat-shock resistant heterotrophs. Bars marked with the same letter are not significantly different ($P \leq 0.0001$). TN, too numerous to count; BD, below detection (< 100 CFU g^{-1}).

Figure 2. Effect of TNT concentration on the growth (A) and TNT transformation ability (B) of gram-positive bacteria. The solid line represents the logistic curve fit to the pooled data for all gram-positive organisms (n = 13) by using nonlinear regression.


A
Uncontaminated
TNT-Contaminated
CFU g-1 (x 10⁶)
200
150
100
50
0
a
c
b
c
c
c
c
c
c
c
B
CFU g-1 (x 10⁴)
70
60
50
40
30
20
10
0
TN
b
a
b
a
b
BD
BD
TSA
Mol
Mol/10
Mol/25
Mol/67


A
B
% Maximum OD
% Initial TNT
Initial TNT (μg mL-1)
120
100
80
60
40
20
0
-20
0
10
20
30
40
50
60
70
80