RRB-89-76

RATIONALE FOR INCLUDING METABOLITES IN CHEMICAL TOXICITY BIOASSAY

by

D. Liu, R.J. Maguire,
B.J. Dutka and G.J. Pacepavicius

Rivers Research Branch
National Water Research Institute
Canada Centre for Inland Waters
Burlington, Ontario, L7R 4A6

October 1989
NWRI Contribution #89-159

ABSTRACT

Toxicity and biodegradability are major factors affecting the fate and behavior of an organic contaminant in the environment. In this report, 2,4-dinitrotoluene (DNT), an important industrial chemical, was used as a model toxicant to demonstrate that toxicity and biodegradability are intimately related in determining a chemical's effect and impact on the total ecosystem. DNT was found to be aerobically stable, but could undergo anaerobic biotransformation with the formation of three metabolites. One of the metabolites, namely 2-nitroso-4-nitrotoluene, was extremely toxic to the 6 test bacterial isolates, while the other 2 aminonitro metabolites and the parent compound DNT were much less toxic or nontoxic to the bacterial cultures. It is suggested that the incorporation of some known metabolites into short-term bioassay procedures could increase the reliability of these tests in the impact assessment of toxic chemicals on the environment. In addition, the difference between toxicity data generated from short-term bioassay procedures and those from long-term toxicological studies was also delineated from a biochemical viewpoint.

RÉSUMÉ

La toxicité et la biodégradabilité sont des facteurs importants qu influent sur le devenir et le comportement d'un contaminant organique l'environnement. Dans ce rapport, nous avons utilisé le 2,4-dinitroto (DNT), un important produit chimique industriel, comme toxique modèle, de montrer que la toxicité et la biodégradabilité sont étroitement lié de la détermination de l'effet et de l'impact d'un produit chimique su l'écosystème dans son ensemble. Le DNT est stable, a-t-on constaté, e aérobie, mais il peut, en milieu anaérobie, subir des transformations accompagnées de la formation de trois métabolites. L'un de ces métabo savoir le 2-nitroso-4-nitrotoluène, était extrêmement toxique pour les isolats bactériens utilisés au cours des épreuves biologiques, tandis autres métabolites 2-aminonitrés et le DNT d'origine étaient beaucoup sinon aucunement toxiques pour les cultures bactériennes. L'utilisati certains métabolites connus permettrait peut-être d'améliorer la fiabi épreuves biologiques à court terme lors de l'évaluation de l'impact de produits chimiques toxiques sur l'environnement. De plus, on expose l différence qu'il y a du point de vue biochimique entre les données de obtenues à l'aide d'épreuves biologiques à court terme et celles obten suite d'études toxicologiques à long terme.

MANAGEMENT PERSPECTIVE

This paper represents a continuation of our process research aimed at evaluating the usefulness and limitations of using short-term bioassay procedures in the impact assessment of contaminants on the environment. A nitroaromatic compound, 2,4-dinitrotoluene (DNT), was used as a model toxicant in this study to demonstrate that toxicity and biodegradability are intimately related in determining the effect and impact on the environment of some chemicals. Incorporation of some known metabolites into short-term bioassay procedures are, therefore, recommended to increase the usefulness and reliability of these procedures in the impact assessment of toxic chemicals on the environment.

PERSPECTIVE-GESTION

Cette communication s'inscrit dans le sillage de nos travaux de sur les processus, dont l'objectif est d'évaluer l'utilité et les limites des épreuves biologiques à court terme dans l'évaluation de l'impact des contaminants sur l'environnement. Au cours de cette étude, nous avons utilisé le composé nitroaromatique 2,4-dinitrotoluène (DNT) comme toxique modèle, afin de montrer que la toxicité et la biodégradabilité sont des phénomènes étroitement liés lors de la détermination de l'effet et de l'impact de certains produits chimiques sur l'environnement. Nous recommandons donc d'incorporer certains métabolites connus dans les épreuves biologiques à court terme, afin de rendre ces méthodes plus utiles et plus fiables lors de la détermination de l'impact des produits chimiques toxiques sur l'environnement.

INTRODUCTION

There are approximately 63,000 chemicals in common use (Maugh, 1978) and new ones are continually introduced. A detailed toxicity assay of all existing chemicals by conventional animal tests is both time-consuming and impractical in terms of cost and space. Thus a wide array of short-term bioassays, using bacteria, yeast, protozoa, and algae has been developed for the rapid screening of chemical toxicity (Bitton, 1983; Dutka and Bitton, 1986). While these short-term bioassays are useful for screening out the problematic toxicant, meaningful interpretation of the toxicity data is not easy, particularly when extrapolation has to be extended to a natural ecosystem. The interactions between organisms and toxicants in the environment are extremely complicated. Many processes and mechanisms, such as biodegradation, biotransformation, syngerism, and antagonism could be all involved in these interactions. These processes/mechanisms, in turn, may have a profound effect on the observed overall toxicity. For example, sometimes a chemical's metabolites or degradation products could be even more toxic than its parent compound to the test organisms (Baarschers et al., 1980; NRCC, 1975), thus greatly complicating the impact assessment of a chemical's toxicity on the environment.

Dinitrotoluene (DNT) is an important industrial chemical, with over six hundred million pounds being used in 1980 for the production of polyurethane foams and elastomers (Rickert et al., 1984). A typical technical grade DNT consists of approximately 76% 2,4-DNT and 16% 2,6-DNT (Guest et al., 1982). Interest in the toxicology of DNT has been increasing for the last decade, as this chemical has been

identified as a potent hepatocarcinogen in rats (CIIT, 1979). However, a discrepancy has been observed between the in vitro and in vivo toxicity results for 2,4-DNT (Miralis et al., 1982). In the present study, 2,4-DNT was used as a model toxicant to demonstrate that the inclusion of some known metabolites or biotransformation products in the short-term bioassay procedures may help toxicity data interpretation, thus extending the usefulness of these tests in impact assessment of contaminants on the environment.

MATERIALS AND METHODS

Chemicals

The test chemicals, 2,4-DNT (2,4-dinitrotoluene), 2-NH_2-4-NT (2-amino-4-nitrotoluene), and 4-NH_2-2-NT (4-amino-2-nitrotoluene) were obtained from Aldrich Chemical Co., Milwaukee. After repeated crystallization from acetone, the identity and purity of these compounds were confirmed by thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry. The biotransformation product, 2-NO-4-NT (2-nitroso-4-nitrotoluene) was synthesized via the oxidation of the amine with caro acid (Sandler and Karo, 1971).

Biotransformation of DNT

The anaerobic biotransformation of 2,4-DNT was conducted at room temperature (22^{o}C) and involved the use of 3 cyclone fermentors as described previously (Liu et al., 1984). In brief, one fermentor served as the abiotic control, containing bacterial inoculum, DNT, and mercuric chloride (microbial inhibitor); another fermentor

contained only bacterial inoculum as a cell control; the biotransformation fermentor included both inoculum and DNT. Each fermentor contained 1 L of basal salt medium (0.5 g each of $NaNO_3$ and K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$ and 0.01 g of $FeSO_4.7H_2O$ in 1 L of distilled water), 100 mL of inoculum, with or without the spiked chemical (5 mg of DNT L^{-1}), or $HgCl_2$ (50 mg L^{-1}). A nitrogen gas flow rate of 20 mL min^{-1} was maintained for each fermentor throughout the exposure time of 12 days. The aerobic biotransformation of 2,4-DNT was performed in the same fashion as the anaerobic experiments, with the exception of a longer incubation period (34 days) and the substitution of air for nitrogen gas.

Analysis of DNT

Extraction and determination of DNT in the biotransformation experiments involved the extraction of 50 mL fermentor broth with 4 x 10 mL of dichloromethane. The extracts were transferred into hexane, evaporated to 2.0 mL, and analyzed on a Hewlett-Packard 5730A gas chromatograph fitted with a flame ionization detector and an electron capture detector. The column (2mm x 180 cm) packing was Chromosorb W(AW-DMCS) coated with 3% OV-1, operating at 130-210°C (4°C min^{-1}). The mass spectra of both the metabolically produced and reference compounds were obtained on a Finnigan model 4000 gas chromatograph-mass spectrometer with OV-1 and DB1710 capillary columns, respectively.

Toxicity Assay

The toxicity of 2,4-DNT and its biotransformation products to microorganisms was determined using the resazurin reduction procedure (Liu and Thomson 1983) and the natural bacterial isolates as the test

organisms (Liu, 1985). The test chemicals were first dissolved in DMSO to form stock solutions and their toxicities were assessed at several concentration levels. The results were plotted as "% inhibition" vs log concentrations to generate the IC_{50} value, which was defined as the toxicant's concentration that causes 50% inhibition of the resazurin reduction. A typical toxicity testing solution consisted of 1 mL of bacterial cells; 2.75 mL of fresh growth medium; (250 - X)uL DMSO; X uL stock solution containing the test chemical; and 1 mL of resazurin solution. In all circumstances the DMSO added was kept at a constant level of 250 uL (final volume = 5.00 mL) in all reaction mixtures.

The resazurin test measures the microbial dehydrogenase activity by accepting electrons flowing from the cell's metabolic processes. In the presence of toxicant, the dehydrogenase activity is suppressed, resulting in a reduced electron flowing being available for resazurin reduction. Thus, toxicity as assessed by the resazurin reduction method is actually a measure of rate inhibition (rate of resazurin reduction in the control vs its reduction rate in the test mixture), i.e., the incubation time is not critical as long as no more than 90-95% of the dye in the cell control is reduced.

RESULTS AND DISCUSSION

Since microbial biotransformations may significantly affect a chemical's toxicity through molecular modification (Rickert et al., 1984), our initial approach was to identify the aerobic and anaerobic metabolites of 2,4-DNT biotransformation. DNT was not biotransformed by aerobic bacterial cultures, even after a 34-day incubation in

a cyclone fermentor. However, under anaerobic conditions DNT underwent a sequential reductive biotransformation in the fermentor, resulting in the formation of three major biotransformation products (Figure 1). Within 48 hours, two aminonitro compounds (2-NH_2-4-NT, and 4-NH_2-2-NT) and one nitrosonitro compound (2-NO-4-NT) were found in the anaerobic fermentor broth. However, these biotransformation products disappeared with time, with only trace amounts of the two aminonitro compounds detectable in the fermentor broth after 12 days' incubation. The nitrosonitro compound (2-NO-4-NT) was considerably less stable than the two aminonitro compounds, as evidenced by its transient occurrence in the early stage of 2,4-DNT anaerobic biotransformation (Figure 1).

Attempts to trace the ultimate fate of the three anaerobic biotransformation products were not conclusive. In one experiment the anaerobic biotransformation of DNT was allowed to proceed for 3 weeks. At various time intervals during this three week period the dichloromethane extract of the fermentor broth (50 mL) was concentrated to 2 mL and the absorbance spectrum was taken on a Beckman DB-GT spectrophotometer (Figure 2). Both the day 1 (A_{max} = 360 nm) and day 2 extracts (A_{max} = 367 nm) showed an absorbance spectrum similar to that of the nitrosonitro compound 2-NO-4-NT (A_{max} = 360 nm), while the absorbance spectrum from the day 7 extract (A_{max} = 430 nm) resembled that of the aminonitrotoluene 2-NH_2-4-NT (A_{max} = 430 nm). The strong absorbance in the UV region for all the extracts (day 1-21) implied that the aromatic ring of 2,4-DNT was not broken. When 2,4,6-trinitrotoluene (TNT), an analogue of DNT, was subject to microbial biotransformation,

the aromatic ring was also found not to be cleaved, but the nitro groups on the ring were reduced to amino groups (McCormick et al., 1976; Kaplan and Kaplan, 1982). In general, it appears that the aromatic ring of nitrotoluene compounds is fairly stable towards microbial degradation.

Although in this study the ultimate fate of 2,4-DNT in the anaerobic biotransformation system is uncertain, it is suspected that this compound, after biotransformation, could become conjugated with organic matter such as dead cell debris in the fermentor broth. Such a reaction may lead to the insolubilization or elimination of DNT from the fermentor broth. Carpenter et al (1978) studied the microbial transformation of ^{14}C-labeled 2,4,6-trinitrotoluene in an activated sludge system and found a significant amount of higher-molecular-weight insoluble TNT conjugates in the humin fraction of the sludge. TNT biotransformation under simulated composting conditions had also resulted in the incorporation of a substantial amount of this chemical into the insoluble organic matter fraction (Kaplan and Kaplan, 1982). These findings demonstrate that biotransformation is probably one of the most important processes for the elimination of certain persistent chemicals from the environment.

The acute toxicity of 2,4-DNT and its three anaerobic biotransformation products (2 aminonitro and 1 nitrosonitro compounds) to 6 different bacteria as measured by the resazurin reduction is shown in Figure 3. The dose concentration-response curves typify microbial response to toxic chemicals, in that the enzyme activity is stimulated at low

toxicant concentrations, the hormesis effect (Stebbing, 1982), and inhibited at higher concentration levels (Liu, 1985). For ease of comprehension, Table 1 presents summary results of the acute toxicity effects (IC_{50}) of 2,4-DNT and its three metabolites to the 6 bacterial cultures. The simultaneous use of several bacterial cultures from various environmental sources, in the determination of a chemical's toxicity, has the advantage of being more representative of microbial responses and thus being able to yield more meaningful conclusions. For example, based on the IC_{50} data from Table 1, it can be reasonably concluded that the test parent compound 2,4-DNT and its two aminonitro metabolites (2-NH_2-4-NT, and 4-NH_2-2-NT) all have a low toxicity to bacteria, except 2,4-DNT to culture F. It can be further inferred that for the two aminonitro metabolites, the positions of the nitro group or amino group, relative to the methyl group, appear to be unimportant in determining their toxicity potential.

Perhaps the most important ecotoxicological implication of this study is the unexpected finding that one of the three 2,4-DNT metabolites studied, namely the nitrosonitro toluene 2-NO-4-NT, was a highly toxic compound (IC_{50} = 6-40 ppm) to all six bacterial cultures (Table 1), when compared with the parent compound DNT and the other two aminonitro metabolites. However, this nitrosonitro metabolite was not stable and a loss of its toxicity potential upon storage was noted. Further spectrophotometric analyses of the stock solution (in DMSO) suggested that (Figure 4) the nitrosonitro toluene had reverted to aminonitro toluene (2-NH_2-4-NT), after one week storage at $4^{o}C$. The easy reversion from the unstable nitrosonitro metabolite to the corresponding more

stable aminonitro toluene compound, would not only explain the difficulty to detect/observe the formation of the nitrosonitro metabolite during DNT anaerobic biotransformation, but may also account for the rapid loss of its toxicity upon storage. The instability or reactivity of the nitroso intermediates from the metabolism of 2,4-DNT by intestinal microorganisms from rat, mouse and man had been suggested by Guest et al (1982) as the cause of DNT's toxicity. However, the inability to isolate such intermediates from the reaction mixture had prevented these workers from testing the toxicity of these metabolites. To our knowledge the present study is the first report on the assessment of the acute toxicity of the nitrosonitro toluene intermediate by bacterial tests.

Another important implication of this study to ecotoxicology is that, without knowing or assessing the toxicity potential of a chemical's major metabolites, an underestimation of the impact of a toxic substance on the total environment may occur. This study and literature data (Baarscher et al., 1980; NRCC, 1975) clearly demonstrate that certain chemicals, after metabolism, can become more toxic than their original form. Since most short-term bioassay procedures employ very brief exposure times, the test chemical is not expected to be metabolized or biotransformed by the test organisms. Therefore, the majority of data from short-term bioassays are likely to be uninformative regarding the toxicity of metabolites. The ultimate impact of the test chemical on the total ecosystem could not, therefore, be estimated or deduced from a series of short-term bioassays. On the other hand, a long-term bioassay procedure would be more suitable for assessing

a chemical's ultimate impact on the environment. The very long exposure time employed by a long-term study would normally suffice for the test organism to metabolize or biotransform the toxicant, thus enabling the system to detect/assess the total toxicity effect of the testing chemical, including its metabolites, on the organism. The above finding may partially explain why sometimes there is a discrepancy in toxicity results between short-term and long-term bioassays. The incorporation of some known metabolites, if the situation permits, into short-term bioassay procedures such as carried out in this study, is recommended. This inclusion would, we believe, increase the usefulness of short-term bioassays in impact assessment of toxic chemicals on the environment.

REFERENCES

Baarscher, W.H., A.I. Bharath, M. Hazenberg, and J.E. Todd. 1980. Fungitoxicity of methoxychlor and fenitrothion and the environmental impact of their metabolites. Can. J. Bot. 58:426-431.

Bitton, G. 1983. Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: A review. CRC Crit. Rev. Environ. Control. 13:51-67.

Carpenter, D.F., N.G. McCormick, and J.H. Cornell. 1978. Microbial transformation of ^{14}C-labeled 2,4,6-trinitrotoluene in an activated-sludge system. Appl. Environ. Microbiol. 35:949-954.

Chemical Industry Institute of Toxicology (CIIT). 1979. A twenty-four month study in Fischer-344 rats given dinitrotoluene. Interim report, CIIT No. 327N8, Research Triangle, N.C.

Dutka, B.J., and G. Bitton. 1986. Toxicity Testing Using Microorganisms. CRC Press, Boca Raton, volume 2, 202 pp.

Guest, D., S.R. Schnell, D.E. Rickert, and J.G. Dent. 1982. Metabolism of 2,4-dinitrotoluene by intestinal microorganisms from rat, mouse, and man. Toxicol. Appl. Pharmacol. 64:160-168.

Kaplan, D.L., and A.M. Kaplan. 1982. Thermophilic biotransformation of 2,4,6-trinitrotoluene under simulated compositing conditions. Appl. Environ. Microbiol. 44:757-760.

Liu, D. 1983. Toxicity assessment of chlorobenzenes using bacteria. Bull. Environ. Contam. Toxicol. 31:105-111.

Liu, D., K. Thomson, and A.C. Anderson. 1984. Identification of nitroso compounds from biotransformation of 2,4-dinitrotoluene. Appl. Environ. Microbiol. 47:1295-1298.

Liu, D. 1985. Effect of bacterial cultures on microbial toxicity

assessment. Bull. Environ. Contam. Toxicol. 34:331-339.

Maugh, T.H. 1978. Chemicals: How many are there. Sci. 199:162-163.

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

Mirsalis, J.C., T.E. Hamm Jr, J.M. Sherrill, and B.E. Butterworth. 1982. Role of gut flora in the genotoxicity of dinitrotoluene. Nature. 295-322-323.

National Research Council of Canada (NRCC). 1975. Fenitrothion: The effects of its use on environmental quality and its chemistry. NRCC report No. 14104, Ottawa.

Rickert, D.E., B.E. Butterworth, J.A. Popp. 1984. Dinitrotoluene: acute toxicity, oncogenicity, genotoxicity, and metabolism. CRC Crit. Rev. Toxicol. 13:217-234.

Sandler, S.R., and W. Karo. 1971. Organic Functional Group Preparations. Academic Press, New York, volume 2.

Stebbing, A.R.D. 1982. Hormesis - the stimulation of growth by low levels of inhibitors. Sci. Total Environ. 22:213-234.

Table 1. IC_{50} (ppm) of 2,4-DNT and its biotransformation products to various bacterial cultures.

Chemicals	IC_{50} (ppm)					
	Cultures					
	A	B	C	E	F	G
2,4-DNT	500	300	290	350	11	500
2-NH_2-4-NT	500	270	490	220	500	500
4-NH_2-2-NT	500	290	335	360	500	500
2-NO-4-NT	22	6	7	40	36	36

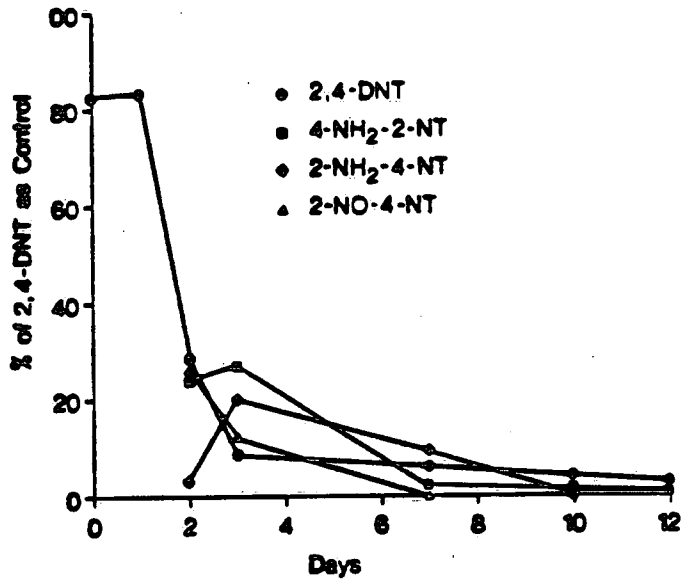
LEGENDS

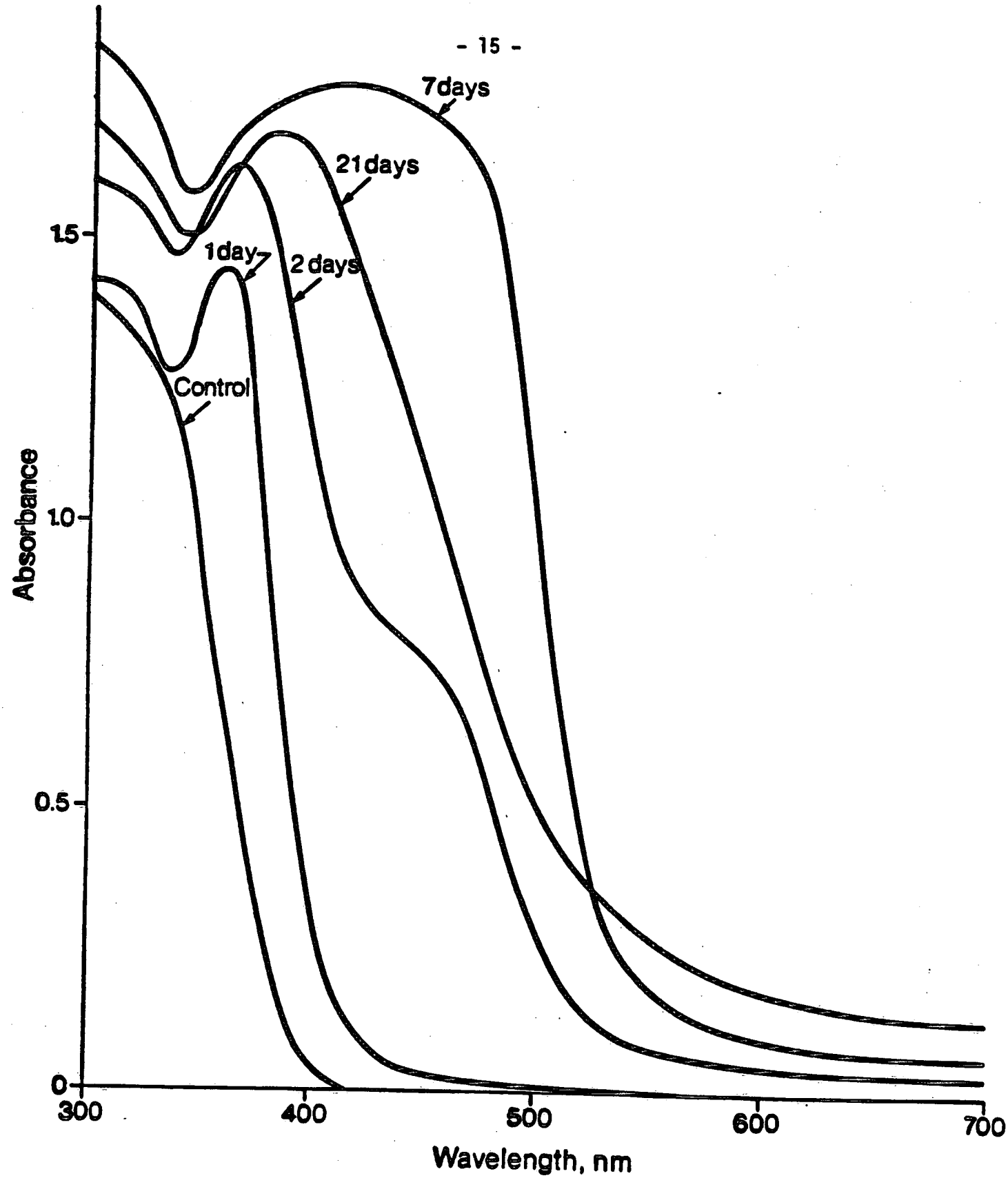
Figure 1. Time course of 2,4-DNT anaerobic biotransformation.

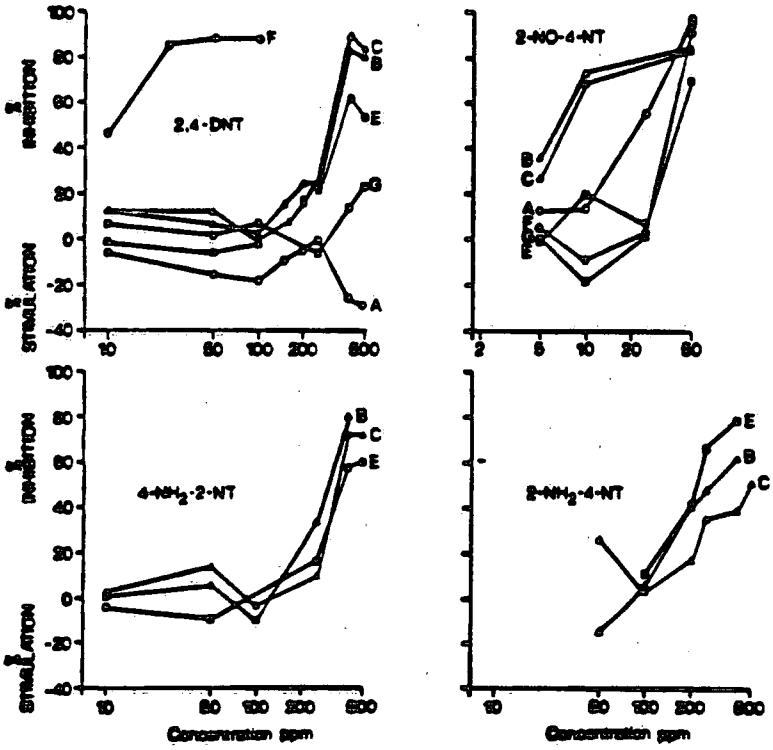
Figure 2. Absorbance spectra of dichloromethane extracts from the anaerobic fermentor broth.

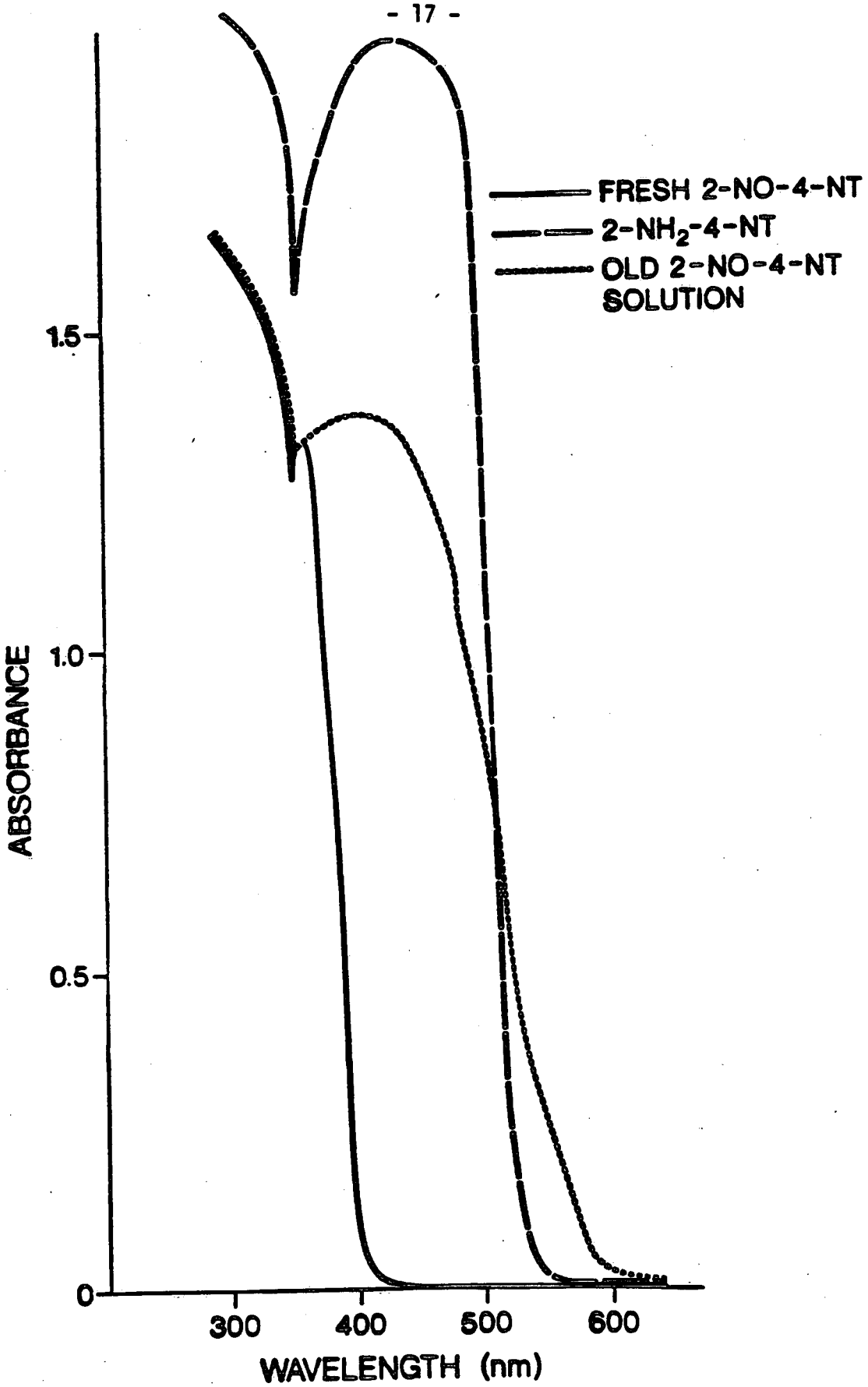
Figure 3. Toxicity of 2,4-DNT and its metabolites to 6 bacterial isolates.

Figure 4. Instability of the nitrosonitro metabolite during storage.


100
80
60
40
20
0
% of 2,4-DNT as Control
0
2
4
6
8
10
12
Days
2,4-DNT
4-NH2-2-NT
2-NH2-4-NT
2-NO-4-NT


7days
21days
1day
2 days
Control
Absorbance
1.5
1.0
0.5
0
300
400
500
600
700
Wavelength, nm


2,4-DNT
2-NO-4-NT
4-NH2-2-NT
2-NH2-4-NT
% INHIBITION
% STIMULATION
Concentration ppm


FRESH 2-NO-4-NT
2-NH2-4-NT
OLD 2-NO-4-NT SOLUTION
ABSORBANCE
1.5
1.0
0.5
0
300
400
500
600
WAVELENGTH (nm)