SPECIAL REPORT 97-32

Evaluation of Commercial Enzyme Immunoassays for the Field Screening of TNT and RDX in Water

Philip G. Thorne and Karen F. Myers

December 1997

Abstract: Water samples from 44 monitoring wells at three military installations were analyzed for the high explosives TNT and RDX using immunoassay test kits. The accuracy and precision of the kit determinations were compared with results obtained using the RP-HPLC, EPA Method 8330. Most of the kits achieved a ±50% relative percent difference criterion over 85% of the time. One of the kits failed this test over half the time. Careful consideration must be given to interferences that may be present and unique for each application.

How to get copies of CRREL technical publications:

Department of Defense personnel and contractors may order reports through the Defense Technical Information Center:

DTIC-BR SUITE 0944
8725 JOHN J KINGMAN RD
FT BELVOIR VA 22060-6218
Telephone 1 800 225 3842
E-mail help@dtic.mil
msorders@dtic.mil
WWW http://www.dtic.dla.mil/

All others may order reports through the National Technical Information Service:

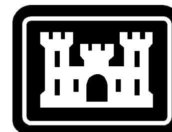
NTIS
5285 PORT ROYAL RD
SPRINGFIELD VA 22161
Telephone 1 703 487 4650
1 703 487 4639 (TDD for the hearing-impaired)
E-mail orders@ntis.fedworld.gov
WWW http://www.fedworld.gov/ntis/ntishome.html

A complete list of all CRREL technical publications is available from:

USACRREL (CECRL-LP)
72 LYME RD
HANOVER NH 03755-1290
Telephone 1 603 646 4338
E-mail techpubs@crrel.usace.army.mil

For information on all aspects of the Cold Regions Research and Engineering Laboratory, visit our World Wide Web site:
http://www.crrel.usace.army.mil

Special Report 97-32



US Army Corps
of Engineers®
Cold Regions Research &
Engineering Laboratory

Evaluation of Commercial Enzyme Immunoassays for the Field Screening of TNT and RDX in Water

Philip G. Thorne and Karen F. Myers

December 1997

Prepared for
U.S. ARMY ENVIRONMENTAL CENTER
SFIM-AEC-ET-CR-97051

Approved for public release; distribution is unlimited.

PREFACE

This report was prepared by Philip G. Thorne, Research Physical Scientist, Geological Sciences Division, Research Directorate, U.S. Army Cold Regions Research and Engineering Laboratory (CRREL), Hanover, New Hampshire, and Karen F. Myers, Biologist, Environmental Chemistry Branch, Environmental Engineering Division, U.S. Army Waterways Experiment Station (WES), Vicksburg, Mississippi.

Funding was provided by the U.S. Army Environmental Center, Aberdeen Proving Ground, Maryland, Martin H. Stutz, project monitor, and the U.S. Army Engineer Waterways Experiment Station, Ann B. Strong, project monitor (AF25-AS-001).

The authors gratefully acknowledge Dr. Paul H. Miyares, CRREL, and Thomas A. Ranney, Science and Technology Corporation, for their technical reviews of this manuscript.

This publication reflects the personal views of the authors and does not suggest or reflect the policy, practices, programs, or doctrine of the U.S. Army or the government of the United States. The contents of this report are not to be used for advertising or promotional purposes. Citation of brand names does not constitute an official endorsement or approval of the use of such commercial products.

CONTENTS

Preface ii
Introduction 1
  Conventional laboratory analyses 1
  Enzyme immunoassay methods for TNT and RDX 1
Experimental methods 2
  Collection of groundwater samples 2
  Laboratory analysis by RP-HPLC 3
  Immunoassay kits 3
Results and discussion 4
  Quantix 8
  EnviroGard 8
  Ohmicron RaPID assay 11
  DTECH 12
Summary and conclusions 13
Further tests and current status 14
Literature cited 14
Abstract 17

ILLUSTRATIONS

Figure
1. Enzyme-linked immunoassay 2
2. Linear ranges of the TNT kits compared with a typical antibody binding curve 3
3. TNT concentration vs. absorbance for four sets of standards using the Quantix kit 9
4. RP-HPLC values vs. average Quantix values 9
5. RP-HPLC values vs. all within-range Quantix values 9
6. EnviroGard TNT plate kit: five sets of standards 10
7. RP-HPLC values vs. average EnviroGard values 10
8. RP-HPLC values vs. all within-range EnviroGard values 10
9. Ohmicron: three sets of standards 11
10. RP-HPLC values vs. Ohmicron average values 11
11. RP-HPLC values vs. all within-range Ohmicron values 12
12. Serial dilutions of well water compared with standard curve 12
13. Standard curve produced at WES vs. the DTECH published curve 13
14. RP-HPLC values vs. DTECH values 13

TABLES

Table
1. Cross-reactivities of TNT kits at the 50% inhibition and detection limit 2
2. Characteristics and performance of test kits 4
3. Samples containing measurable analytes by RP-HPLC 5
4. Samples containing no target analytes as determined by RP-HPLC 8

Evaluation of Commercial Enzyme Immunoassays for the Field Screening of TNT and RDX in Water

PHILIP G. THORNE AND KAREN F. MYERS

INTRODUCTION

Contaminated groundwater on and around U.S. military installations is a serious problem. Besides the usual toxic and hazardous materials common to many large industries, the military is a unique source of nitroaromatic and nitramine secondary explosives. The EPA has set low allowable intake limits for these classes of compounds in drinking water (EPA 1988a,b,c, 1989, 1992). To comply with EPA guidelines and to protect the public safety, the U.S. military must identify and remediate sources of contamination.

Conventional laboratory analyses

The traditional approach used to delineate the extent and degree of explosives contamination has been to analyze monitoring well samples at off-site laboratories using SW846 Method 8330 (EPA 1994). Off-site analysis requires several days' turnaround and delays on-site decision making. Site managers cannot optimize sampling strategies without data on initial samples. In addition, laboratory analyses generally cost over $200 per sample. This cost limits the number of samples that can be analyzed, decreasing the spatial resolution of the investigation. A survey of results from 46 military installations (Walsh et al. 1993) showed that approximately two-thirds of the soil samples and three-quarters of the water samples analyzed were not contaminated with secondary explosives or transformation products. Of the samples that were contaminated, 95% contained TNT (2,4,6-trinitrotoluene) and/or RDX (1,3,5-hexahydro-1,3,5-trinitrotriazine). Significant cost reductions can be achieved if negative samples could be identified without off-site analysis. Furthermore, rapid field determination of the degree of contamination, where present, can assist site managers in prioritizing their sampling efforts.

Field methods for the detection and quantification of TNT and RDX in soil and water have been developed that rely on the colorimetric analysis of highly specific reaction products (Jenkins and Walsh 1992, Jenkins et al. 1994). The detection limits for these tests are 1 μg/g TNT and RDX in soil and 0.9 μg/L TNT and 3.8 μg/L RDX in water. Several samples an hour can be processed using these methods.

Enzyme immunoassay methods for TNT and RDX

Enzyme immunoassays are analytical methods based on highly selective binding reactions of antibodies with specific target analytes. Antibodies are proteins produced in response to foreign substances as part of the vertebrate immune response system. Methods developed for small molecules are usually formatted as competitive enzyme-linked immunosorbent assays (ELISAs). In one common form of ELISA (Fig. 1), the target analyte is bound to an enzyme through a spacer molecule to form an enzyme–analyte conjugate. Antibodies are bound onto the surface of a solid, such as the walls of a microtitre well or test tube, or onto small spheres. When a known amount of enzyme–analyte conjugate and sample containing the free target analyte are mixed with the antibodies, they compete with each other for binding sites on the antibodies. Upon the addition of the appropriate substrate, the enzyme catalyzes a reaction that changes a chromogen from colorless to colored. Quantitation is accomplished by comparing color intensity to a standard curve. The

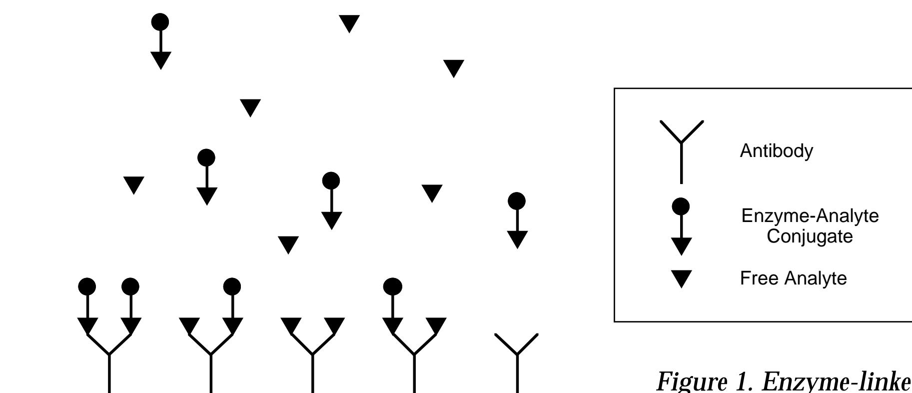


Figure 1. Enzyme-linked immunoassay.

amount of enzyme conjugate retained on the antibodies (i.e., color change) is inversely proportional to the amount of target analyte in the sample (Vanderlaan et al. 1990). In other words, the more intense the color development, the lower the concentration of free analyte in the sample. A less intense color indicates higher concentrations of free analyte.

Many environmental contaminants are small molecules that cannot induce antibody production by themselves. These molecules must be covalently bound to larger carrier proteins in order to stimulate antibody production when injected into an animal. These small molecule–protein conjugates are called haptens. The specificity of an antibody to a target analyte can be influenced by the design of the hapten. This is done by controlling the orientation and spacing between the analyte and carrier protein used to induce the immunological effect. Through careful selection of antibodies it is possible to design immunoassays that can distinguish an analyte from a related family of compounds or a parent compound from its metabolites (Keuchel et al. 1992a,b). In the case of TNT, conjugates could be made by coupling a protein to either a reactive moiety at the 1- position (e.g., trinitro-sulphonic acid) or at the 2- or 4- position (2- or 4-aminodinitrotoluene). The antibody would then tend to recognize either a trinitro-aromatic or a dinitro-toluene, respectively. The binding of antibodies to non-target analytes is termed cross reactivity. For molecules with limited numbers of functional groups, specificity becomes more difficult and cross reactivity with other structurally related molecules becomes more likely.

The various schemes that were used by the four manufacturers to produce anti-TNT antibodies resulted in a wide variety of cross-reactive analytes and relative degrees of interference (Table 1). In general, manufacturers emphasize the degree of cross-reactivity that occurs in the middle of the

Table 1. Cross-reactivities of TNT kits at the 50% inhibition (midrange) and detection limit (μg/L).

Analyte	*EnviroGard 50%/DL*	*Ohmicron 50%/DL*	*DTECH 50%/DL*	*Quantix 50%/DL*
TNT	3/0.5	1.44/0.07	22/5	1.00/0.05
TNB	95/6	2.20/0.04	96/20	0.47/*
2ADNT	>1000/1.6	45/0.25	200/30	<0.05/*
4ADNT	16/0.7	98/0.10	>500/>500	0.02/*

*Quantix could not supply the DL cross-reactivities.

range of their kits (IC_{50}). However, the nature of antibody–hapten interaction produces a curve of concentration vs. binding that is sigmoidal (Fig. 2). Thus, at concentrations close to the detection limits of each kit, the cross reactivities tend to be more pronounced. Environmental degradation products of TNT, such as TNB and amino-DNTs, can produce a significant additional response.

Inaccurate responses in immunoassays can also be caused by compounds that disrupt either antibody binding or enzyme activity. This phenomenon is called interference. In either case, the amount of color development will be less than anticipated—i.e., a false positive response. This is a better choice than a test that would result in false negatives due to environmental interferences. All of the kits tested reduce potential interferences by diluting the sample in an assay solution containing a buffer and bovine serum albumin to reduce the effects of extreme pH and humic materials (Keuchel et al. 1992c).

DTECH produces the only immunoassay for RDX. Its cross reactivity to HMX is only 3% at the detection limit. It has no response to nitroaromatics.

EXPERIMENTAL METHODS

Collection of groundwater samples

Groundwater samples were collected from 33 monitoring wells at the Naval Surface Warfare

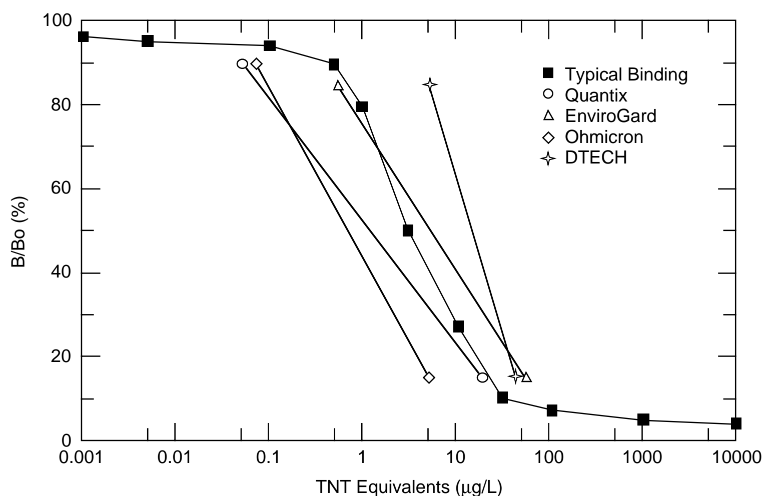


Figure 2. Linear ranges of the TNT kits compared with a typical antibody binding curve. % B/Bo *= (Absorbance of sample/Absorbance of negative control) × 100.*

Center in Crane, Indiana. Bailers used for collections were rinsed once with isopropyl alcohol and three times with distilled water between samples. Wells were purged with a PVC bailer to a depth midway down the well, allowed to recharge for 2 hours, then sampled with the cleaned Teflon bailers. Samples were collected in 1-liter precleaned, amber glass bottles and were stored and shipped at 4°C (39°F). Samples from five monitoring wells at Umatilla Army Depot Activity, Oregon, and six wells at the U.S. Naval Submarine Base, Bangor, Washington, were supplied by Black and Veatch Waste Science, Tacoma, Washington.

Laboratory analysis by RP-HPLC

Water samples were analyzed as described in SW846 Method 8330 (EPA 1994). At WES, samples were diluted 1:1 with methanol, and aliquots of 50 µL were injected on two RP-HPLC systems (Millipore/Waters Chromatography Division, Milford, Mass.). The primary system consisted of a Waters model 600E MS System Controller, a 712 WISP Auto Injector, a 486 UV Variable-Wavelength Detector monitored at 254 nm, and a Maxima 820 chromatography workstation. The columns used were Supelco 25 cm × 4.6 mm LC-18 for the primary separation and 25 cm × 4.6 mm LC-CN for the confirmation separation. Analytes were eluted with 1:1 methanol/water at flow rates of 1.0 mL/min and 1.2 mL/min, respectively. Water samples were also concentrated by salting-out solvent extraction (SOE) and analyzed on the same systems. Stored samples were analyzed at CRREL, using identical columns and 10-µL aliquots of 1:1 methanol-diluted samples. The chromatography system consisted of a Spectra Physics 8810 pump, 8875 autosampler, and 8490 detector at 254 nm. Peak heights were recorded with a Hewlett-Packard 3396 Integrator.

Immunoassay kits

Kits from four manufacturers were tested for this report. Although each kit was based on the same principals of a competitive immunoassay, each kit was formatted differently and had widely different dynamic ranges (Table 2).

The EnviroGard (Millipore, Medford, Mass.) and Quantix (Idetek, Sunnyvale, Calif.) kits are intended as quantitative laboratory assays, although they can be implemented in field situations with battery-powered spectrophotometers. The antibodies were immobilized onto the surfaces of 96-well microtitre strip-plates. Duplicate water samples or standards and enzyme-conjugated TNT were diluted in an assay buffer in the wells of the plate. These were incubated at room temperature for either 60 minutes in the EnviroGard kit or 30 minutes in the Quantix kit. The wells were then rinsed to remove unbound free and conjugated TNT. The substrate and chromogen were added and a blue color allowed to develop for 30 minutes. An acid solution was added to stop the enzyme reaction and change the color of the chromogen from blue to yellow. The absorbance was measured with a spectrophotometer designed to read microtitre strips. Concentrations of analytes were calculated by semi-log regression

Table 2. Characteristics and performance of test kits.

Kit	*Initial cost ($)*	*Format*	*Range (µg/L)*	*Kit cost ($)*	*Cost/day (10 samples)*	*% false neg/pos*	*Accuracy %<RPD = 50*
Quantix	6300 (4000 for govt.)	96-well strips	0.05–20	840	210	0/0	100
EnviroGard	2130	96-well strips	0.5–50	387	97	0/6	86
Ohmicron	4435	Test tubes	0.07–5.0	210	168	0/0	85
DTECH TNT	0	Cups	5-45	100	250	0/30	58
DTECH RDX	0	Cups	5-45	100	250	24/18	32

against the standards by programs within the Quantix-supplied reader.

The RaPID kit (Ohmicron, Newtown, Pa.) is a quantitative laboratory assay that can be utilized in the field with a battery-powered spectrophotometer. Antibodies were immobilized on plastic beads containing a ferrous metal particle. Duplicate samples or standards, TNT conjugate, antibody beads, and diluent were incubated in 12-mm × 75-mm plastic test tubes for 15 minutes. The tubes were then placed in a rack that contained strong magnets. The particles were drawn to the sides of the tube where they remained when the liquid contents of the tube were poured off and the particles rinsed. Substrate and chromagen was then added and the color developed for 20 minutes. Absorbances were measured and concentrations calculated against the standards by the Ohmicron-supplied spectrophotometer.

The DTECH TNT and RDX kits (EM Science, Gibbstown, N.J.) are semiquantitative field tests that require no electronic equipment. Antibodies were immobilized on plastic beads contained in small vials. For each test, a sample diluted in assay buffer containing TNT conjugate was added to one vial and buffer containing only the TNT conjugate was added to a second vial. These incubated for 2 minutes. Then the vials were swirled to suspend the particles and the contents were poured into side-by-side wells in the top of a cup. The bottom of each well was constructed of porous material that allowed the liquid contents of the vial to drain into absorbent material in the cup while retaining the antibody-coated beads. The beads were rinsed in place and substrate was added along with a chromogen that produced a blue precipitate upon activation by the conjugated enzyme. Concentration ranges were determined by comparing the color of the sample well to the color on a test card after the color of the reference well had reached a sufficient intensity to match the reference color on the card. The time required for development depends on temperature and was predicted to be around 10 minutes. Alternatively, a differential reflectometer supplied by DTECH could be used to quantify the inhibition due to TNT or RDX in the sample compared with the reference. The resulting number is then converted to a concentration range based on a calibration table supplied with the kit.

RESULTS AND DISCUSSION

Results from the RP-HPLC analyses showed that 19 of the 33 Crane wells were contaminated with nitramine and nitroaromatic explosives and their environmental transformation products (Tables 3 and 4). The samples that were stored at CRREL for one month were only analyzed for TNT and its transformation products by direct injection. Some of the samples had concentrations that dropped below the detection limit of this method. Other samples showed significant transformation of TNT. All of the Umatilla and Bangor wells had detectable levels of nitramines and nitroaromatics.

There are two ways of evaluating the TNT results from each kit. One way assesses the ability of the kits to determine correctly if there is contamination above the EPA's health advisory limit of 2 µg/L (EPA 1989). The DTECH kit has a detection limit above that and could not be assessed by this criterion. All of the other TNT kits were successful in indicating the presence of TNT when it was there at greater than 2 µg/L. There were no false negatives. They could be used in remediation projects to indicate when contamination levels dropped below the detection limit of the kit.

Another way to assess kit performance is to measure accuracy using the relative percent difference (RPD), where

Table 3. Samples containing measurable analytes (μg/L) by RP-HPLC. False negatives and false positives are in bold. Stored samples were analyzed at CRREL.

Well no.	*pH*	*Type*	*HMX*	*RDX*	*TNB*	*DNB*	*DNA*	*TNT*	*ΣTNT**	*DNT*	*2ADNT*	*4ADNT*
CRANE												
615	7.1	DIR	<0.2 all analytes									
		SOE	1.45	2.45				0.47			0.36	0.32
		DTECH			5–15			5–15	0.52			
		OHM			0.74	0.79						
		EG			<0.5	0.53						
616	7.1	DIR	94.0	79.0								
		SOE	54.2	63.8				0.26	0.33		3.08	1.36
		DTECH		25–45				<5	0.79			
		OHM						1.58	1.07			
		EG						2.9	0.60			
		storedDIR						<2.0			<2.0	<2.0
		EG						2.5				
		QTX						<0.05				
617	6.8	DIR	93.0	91.0								
		SOE	85.7	75.3			0.22	0.19	0.08		2.43	1.31
		DTECH		25–45				<5	0.45			
		OHM						0.6	0.65			
		EG						2.0	0.68			
		storedDIR						<2.0			<2.0	<2.0
		EG						2.0				
		QTX						<0.05				
618	6.8	DIR	45.0	14								
		SOE	45.7	16.4			0.17				2.18	1.21
		DTECH		5-15					<5	0.42		
		OHM							0.36	0.47		
		EG							4.5	0.24		
		storedDIR							<2.0		<2.0	<2.0
		EG							3.1			
		QTX							<0.05			
618	7.0	DIR	45.0	11.0								
Dupl		SOE	45.5	14.1			0.09				1.82	1.08
		DTECH	5-15						<5	0.27		
		OHM							0.32	0.45		
619	6.9	DIR	<0.2 all analytes									
		SOE	0.76	5.77							0.13	
		DTECH		**<5**					<5	0.02		
		OHM							<0.07	<0.07		
		EG							<0.5	<0.5		
622	7.2	DIR	134	365								
		SOE	75.4	202					0.98		8.12	1.80
		DTECH	150-250						<5	2.18		
		OHM							4.5	1.63		
		EG							1.4	1.67		
623	7.0	DIR	<0.2 all analytes									
		SOE	0.61	10.9								
		DTECH		**<5**					<5			
		OHM							<0.07			
		EG							<0.5			
624	7.1	DIR	25.0	13.0								
		SOE	30.2	12.1								
		DTECH		5-15					<5			
		OHM							0.1			
		EG										

* ΣTNT = the sum of TNT equivalents based on the particular cross reactivities for each kit.
Note: DIR = direct injection, SOE = salting-out extraction, OHM = Ohmicron, EG = EnviroGard, QTX = Quantix, Dupl. = duplicate.

Table 3 (cont'd). Samples containing measurable analytes (μg/L) by RP-HPLC.

Well no.	*pH*	*Type*	*HMX*	*RDX*	*TNB*	*DNB*	*DNA*	*TNT*	*ΣTNT*	*DNT*	*2ADNT*	*4ADNT*
646	—	DIR	<0.2 all analytes									
		SOE	1.05	6.17							0.71	0.33
		DTECH		5-15				<5	0.13			
		OHM						0.4	0.21			
		EG						<0.5	0.09			
651	—	DIR	<0.2 all analytes									
		SOE	0.33	7.12								
		DTECH		**<5**				<5				
		OHM						0.44				
		EG						<0.5				
713	6.4	DIR		13.0								
		SOE		5.98								
		DTECH		**<5**				**5–15**				
		OHM						0.08				
		EG						<0.5				
717	6.2	DIR		40.0								
		SOE	0.58	28.7			0.04			0.39	0.13	
		DTECH	**<5**					<5	0.06			
		OHM						<0.07	0.12			
		EG						0.7	0.04			
725	6.0	DIR	165	58.0							9.00	7.00
		SOE	141	39.1			0.79	0.96			8.5	5.62
		DTECH		**<5**				5-15	1.7			
		OHM						4.1	1.54			
		EG						22	1.4			
727	6.4	DIR	173	76.0				17.0			59.0	54.0
		SOE	172	69.5			2.59	23.1		1.2	65.2	56.4
		DTECH		**<5**				15-25	29			
		OHM						29.2	22.4			
		EG						51	27.8			
		storedDIR					10.5	18.0			30.0	30.0
		EG						53	25.5			
		QTX						11	18.8			
731	6.4	DIR	252	157	5.00			110			47.0	65.0
		SOE	227	132	6.62			102			42.6	56.5
		DTECH		**<5**				150-250	118			
		OHM						176	115			
		EG						165	123			
		storedDIR			13.7		15.6	115			25.0	50.0
		EG						145	118			
		QTX						114	111			
733	6.5	DIR	218	40.0								
		SOE	201	35.9							2.2	1.9
		DTECH		5–15				<5	0.29			
		OHM						0.28	0.60			
		EG						2.0	0.38			
737	6.1	DIR	<0.2 all analytes									
		SOE	2.15	7.54								
		DTECH		**<5**				**5–15**				
		OHM						<0.07				
		EG						<0.5				
743	4.5	DIR	112	608	8.00			180			10.0	8.00
		SOE	82.8	429	4.45	0.79		137			7.71	6.20
		DTECH		125–225				750–1250	182			
		OHM						270	185			
		EG						206	182			
		storedDIR			9.2			179			6.3	6.3
		EG						176	181			
		QTX						124	180			

Table 3 (cont'd).

Well no.	*pH*	*Type*	*HMX*	*RDX*	*TNB*	*DNB*	*DNA*	*TNT*	*ΣTNT*	*DNT*	*2ADNT*	*4ADNT*
745	5.8	DIR	325					14.0			51.0	40.0
		SOE	290	87.5	6.31			13.9			42.3	33.5
		DTECH		25–75				25–45	22.7			
		OHM						34.5	18.7			
		EG						114	22			
		storedDIR					8.3	7.6			27.6	13.6
		EG						82	18			
		QTX						11.6	8.0			
2 ppb Blank spike												
		DTECH		5–15				5–15				
		OHM						4.30				
		EG						2.3				
UMATILLA												
4-1		DIR	1290	2370	241				1993			
		OHM						2685	2165			
4-18		DIR	1850	1880	316				3627			
		OHM						3620	3848			
4-P4		DIR	81	4500	41				2			
		OHM						111	28			
4-103		DIR	51	1520	4.2				2			
		OHM						6.9	5			
4-7		DIR	219		0.3							
		OHM						1.44	0.2			
BANGOR												
BEW1		DIR	151	539	151			315				
		OHM						481	413			
BEW2		DIR	35	678	13			30				
		OHM						76	38			
BEW4		DIR	39	2.7								
		OHM						0.56	1.7			
PRE1-6		DIR	31	285	23			46				
		OHM						159	61			

$$\text{RPD} = \frac{(\text{kit value} - \text{lab value})}{(\text{kit value} + \text{lab value})/2} \times 100.$$

A ±50% RPD is the commonly used control limit for field kits. The EnviroGard, Quantix, and Ohmicron kits met this criterion more than 85% of the time. The DTECH kits failed this criterion over half the time. Positive results require confirmation by laboratory analysis. In on-site assessment, false positives add to the cost of laboratory assays. In remediation activities, false positives could become quite expensive if they triggered an additional treatment step (e.g., replacing a purification cartridge or extending a composting period or excavating an additional lift of soil). For the discussion of results that follows, a false positive is defined as an erroneously high value for TNT contamination above the EPA 1989 Health Advisory of 2 µg/L.

The RDX kit produced very poor results for these samples. It failed to detect RDX in eight of the samples that contained RDX above the EPA Health Advisory limit of 2 µg/L (EPA 1988a). It also produced six false positives.

The cost of the kits requiring daily calibration varies depending on the number of samples analyzed per day. The more samples that can be analyzed, the better. For this investigation of monitoring wells at NSWC, 33 samples were generated in 3 days. For cost comparison estimates, ten sample analyses per day was chosen. The strip-plate kits have a unique problem that becomes apparent because of their design. There are 96 assay wells arranged in long strips containing either 12 (EnviroGard) or 8 (Quantix) wells each. All wells in a strip must be used. Unused well-strips cannot be saved for future use. Thus 10 samples, one standard, and one blank run in duplicate (24

Table 4. Samples containing no target analytes (<0.2 μg/L) as determined by RP-HPLC. False positives listed in bold.

Well no.	*pH*		*RDX*	*TNT*
625	—	DTECH	<5	<5
		OHM		<0.07
		EG		<0.5
627	—	DTECH	<5	<5
		OHM		<0.07
		EG		<0.5
715	6.6	DTECH	**5-15**	<5
		OHM		<0.07
		EG		<0.5
719	4.5	DTECH	<5	<5
		OHM		<0.07
		EG		<0.5
729	6.1	DTECH	<5	**5-15**
		OHM		<0.07
		EG		<0.5
735	5.9	DTECH	**5-15**	**5-15**
		OHM		<0.07
		EG		**1.2**
739	5.8	DTECH	**15-25**	**5-15**
		OHM		<0.07
		EG		<0.5
741	3.7	DTECH	<5	**5-15**
		OHM		<0.07
		EG		0.7
766	5.7	DTECH	<5	**5-15**
		OHM		<0.07
		EG		<0.5
		storedQTX		<0.05
768	3.9	DTECH	<5	**5-15**
		OHM		<0.07
		EG		<0.05
		storedQTX		<0.05
770	5.3	DTECH	**5-15**	**5-15**
		OHM		<0.07
		EG		<0.5
		storedQTX		<0.05
772	4.7	DTECH	**5-15**	<5
		OHM		<0.07
		EG		<0.5
774	—	DTECH	**5-15**	**5-15**
		OHM		<0.07
		EG		<0.5
Lab blank		DTECH	<5	<5
		OHM		<0.07
Field blank		DTECH	<5	<5
		OHM		<0.07
		EG		<0.5
BEW5	—	OHM	82	0.39
BEW6	—	OHM	852	1.36

wells) require two EnviroGard strips or three Quantix strips. An eleventh sample would require the use of an additional strip.

These kits have other potential uses, and other problems, that are best discussed with reference to each kit's characteristics.

Quantix

The Quantix kit is primarily intended as a quantitative laboratory assay. It is the most expensive and complicated of the four kits. A strip-reader, an orbital plate-shaker for incubations, a plate-washer, and wash solution were recommended to achieve the best results. Five standards and a negative control were supplied for calibrations. The Quantix strip-reader has a program that calculates results based on a five-point calibration. The linearity and reproducibility of four standard curves was excellent, with a relative standard deviation of slopes equal to 4.4% (Fig. 3). However, a plot of the accuracy of the determinations (Fig. 4) reveals an underestimation of concentrations compared with the RP-HPLC determinations. Two of the samples required a 1:100 dilution with reagent-grade water to fall within the range of the test. If the results of the diluted samples are compared with a predicted $^1/_{100}$ value of the RP-HPLC determination and the data regressed, the accuracy is better (Fig. 5). The scatter in the data is then more obvious. The RPD criterion was met 100% of the time. This was the only kit tested that produced no false positives at its detection limit.

EnviroGard

The EnviroGard kit is also intended as a quantitative laboratory assay. The EnviroGard kit requires only a strip-reader for quantitation. A plate-shaker is listed as optional, as are plate-washers and wash buffers other than tap water. One vial of TNT standard concentrate is supplied and must be diluted to make the suggested three standards. A negative control is supplied. The manufacturer recommends that absorbances be normalized against the negative control and expressed as a percent inhibition (%B/B_o, where B = absorbance of sample and B_o = absorbance of negative control). The calculation of five standard curves produced a relative standard deviation of slopes equal to 4.0% (Fig. 6). A negative control and one standard would probably suffice for routine tests. The calculated concentrations were low compared with the RP-HPLC concentrations of

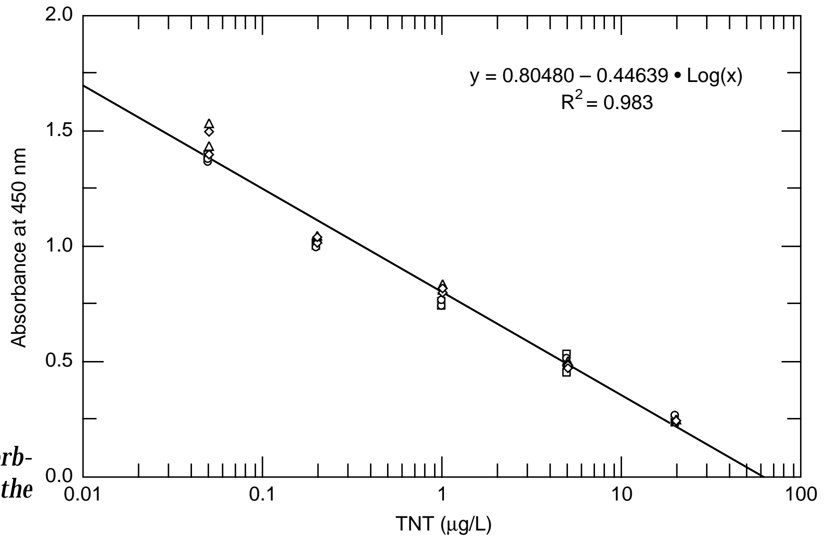


Figure 3. TNT concentration vs. absorbance for four sets of standards using the Quantix kit.

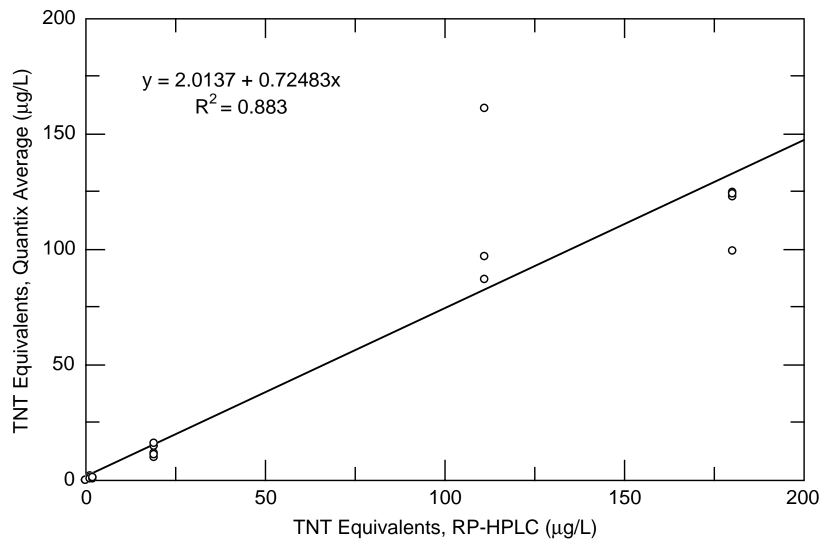


Figure 4. RP-HPLC values vs. average Quantix values.

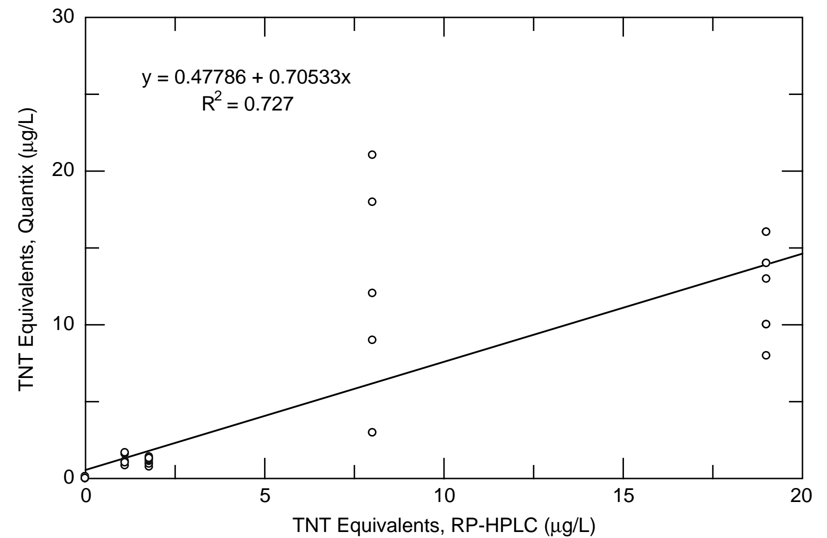


Figure 5. RP-HPLC values vs. all within-range Quantix values

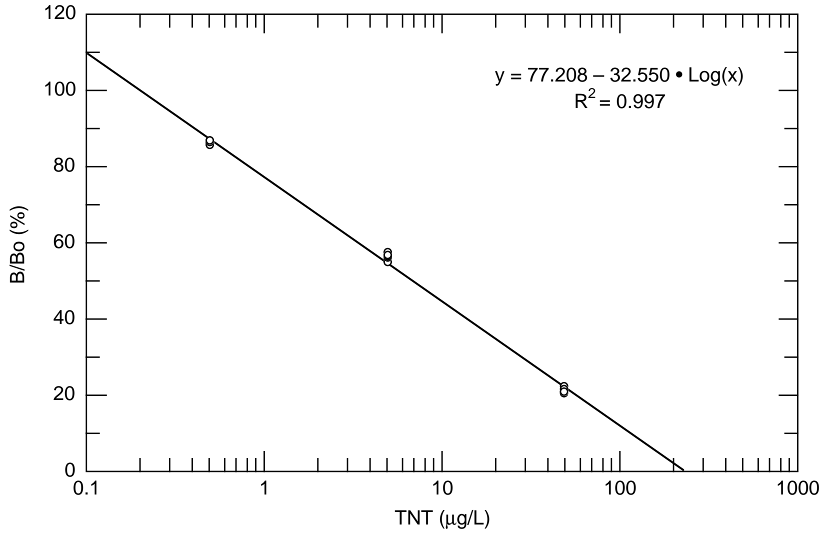


Figure 6. EnviroGard TNT plate kit: five sets of standards.

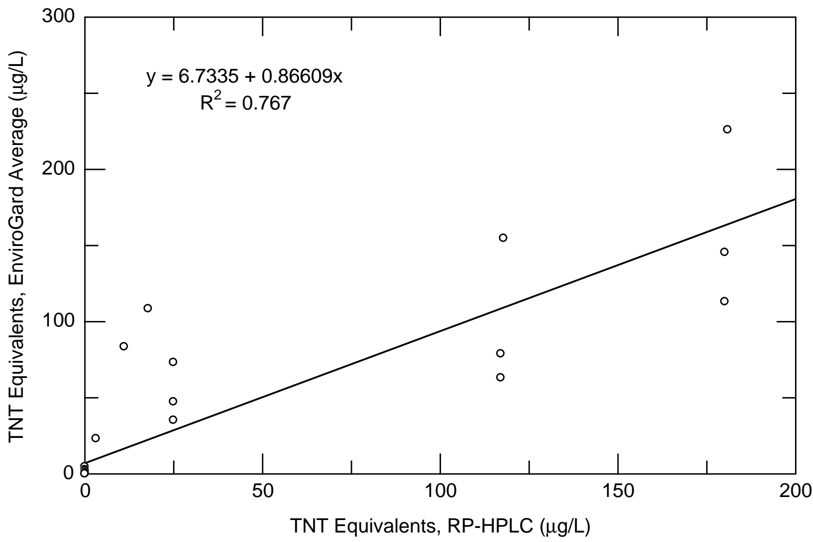


Figure 7. RP-HPLC values vs. average EnviroGard values.

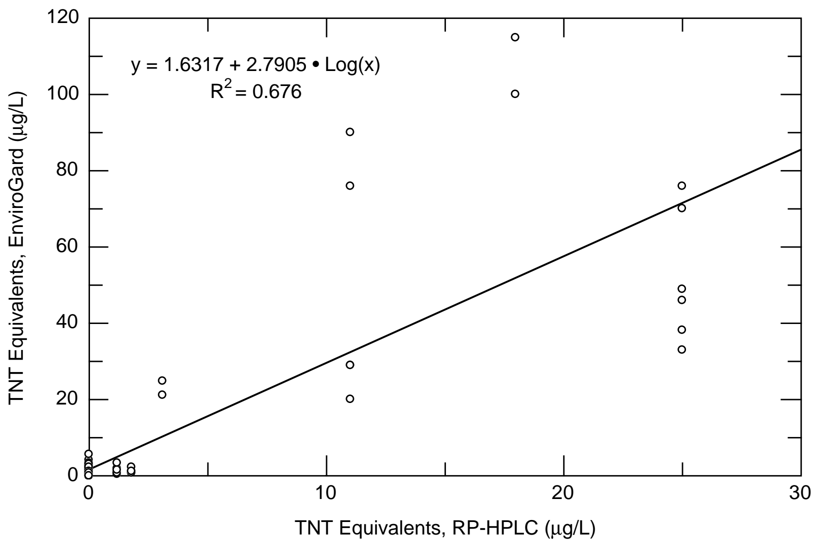


Figure 8. RP-HPLC values vs. all within-range EnviroGard values.

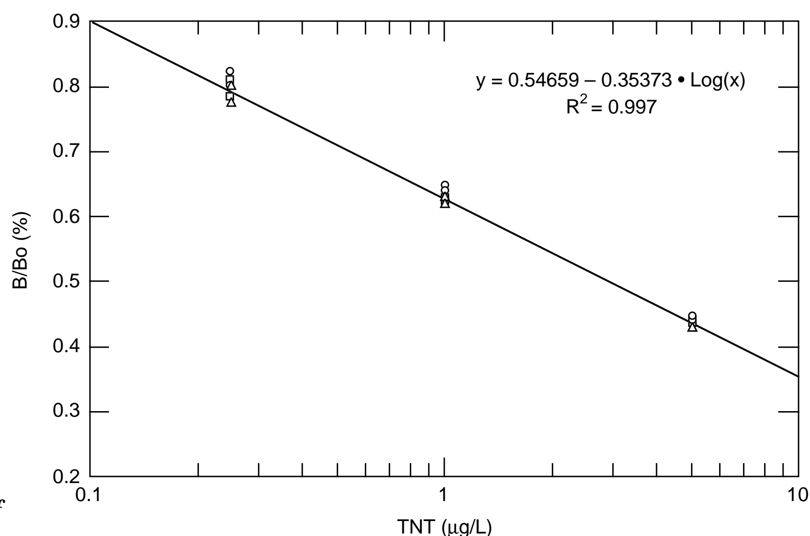


Figure 9. Ohmicron: three sets of standards.

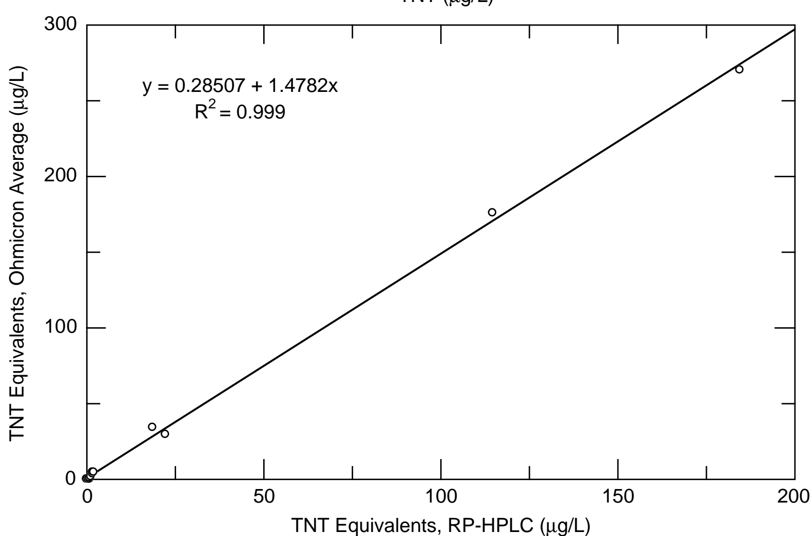


Figure 10. RP-HPLC values vs. Ohmicron average values.

TNT when the regressed comparison extended to the three samples that needed dilution to fall within the range of the kit (Fig. 7). The RPD criterion was met 86% of the time. An examination of the individual values produced within the range of the kit (Fig. 8) shows a large overestimation. This overestimation of concentrations produced two false positives. These samples did however contain several µg/L of TNT transformation products. If there are eight or more samples a day to analyze, this kit is the least expensive to use; however, the cost of false positives must be carefully considered.

Millipore also offers a TNT in Soil kit that uses test tubes and a standard spectrophotometer for quantitation. Since its detection limit is around 2 µg/L, it is not offered for sale as a water test kit. *

Ohmicron RaPID assay

The Ohmicron kit is intended to be a quantitative replacement for Method 8330 RP-HPLC determination. It includes a negative control, prediluted standards, and a check solution, whose value should lie within a range of accuracy in order to proceed with the test. Three calibration curves based on the percent inhibition compared with the negative control had a relative standard deviation of slopes equal to 3.4% (Fig. 9). A comparison between the RP-HPLC concentrations and the average calculated values and all of the within-range, diluted values (Fig. 10 and 11) shows that this kit overestimated the level of contamination. The RPD criterion was met 85% of the time. No false positives occurred above the 2-µg/L level. Three of the Crane wells that required dilution to fall within the range of the kit were assayed at two different dilutions. When the slopes of these serial

* Personal communication, B. Furgeson, Immunosystems, Scarboro, Maine, 1994.

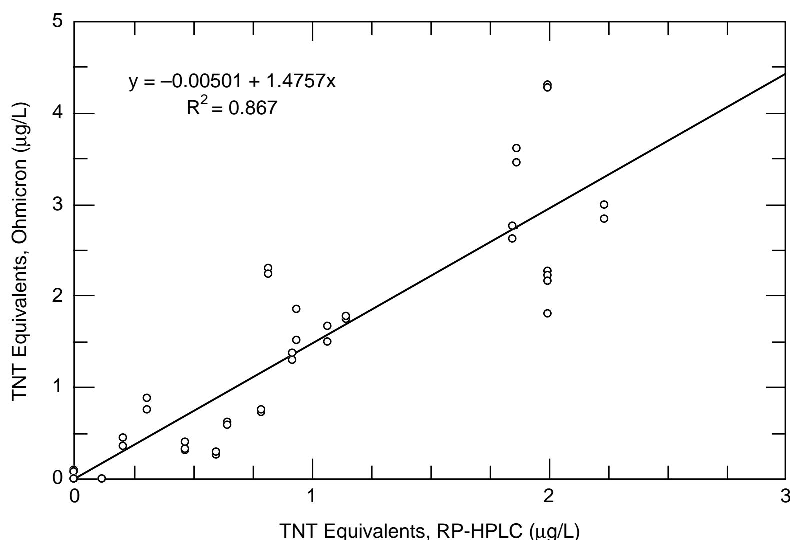


Figure 11. RP-HPLC values vs. all within-range Ohmicron values.

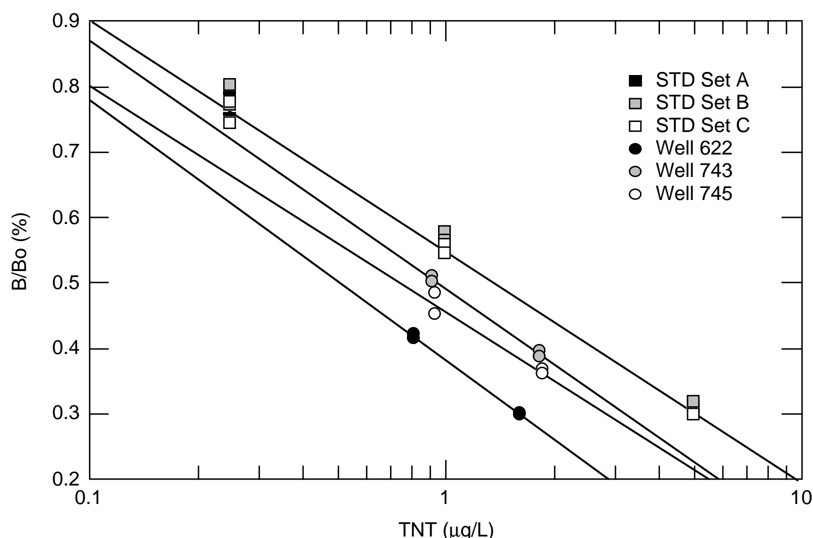


Figure 12. Serial dilutions of well water compared with standard curve.

dilutions are compared with the slope of the calibration curve (Fig. 12), little or no matrix effect was apparent. The wells from Umatilla and Bangor did appear to have a positive matrix interference. The most accurate determinations were achieved when samples required substantial dilution with deionized water to fall within the range of the kit. The largest deviations from RP-HPLC values occurred when samples were analyzed without dilution. The cost of this kit is intermediate and could be lowered approximately 50% by buying the assay tubes in bulk and running only one standard and one negative control each assay.

DTECH

The DTECH kits are small and completely self-contained field kits. The materials for four samples could be carried in a large coat-pocket. They do not include provisions for producing a standard curve. Each test runs with its own reference standard to mark the end-point of the color development. Duplicate analyses require another matching reference standard. They also produce the quickest analyses: 10 minutes vs. 45 to 90 minutes for the other kits. The use of concentration ranges for quantitation emphasizes the qualitative nature of the analyses. Although not required, a TNT standard curve was produced using the numerical values from the reflectometer (Fig. 13). The standard curve published with the kit is quite different. The accuracy and precision of the analyses are plotted with references to the ranges specified on the color card and reflectometer conversion chart. In all cases, the reflectometer and color card agreed. The ranges are filled in with frequency of occurrence values (Fig. 14). Except for one value in the 25–45-µg/L range, the kit overestimated the RP-HPLC values. Multiplying the range as detected by the dilution factor produced

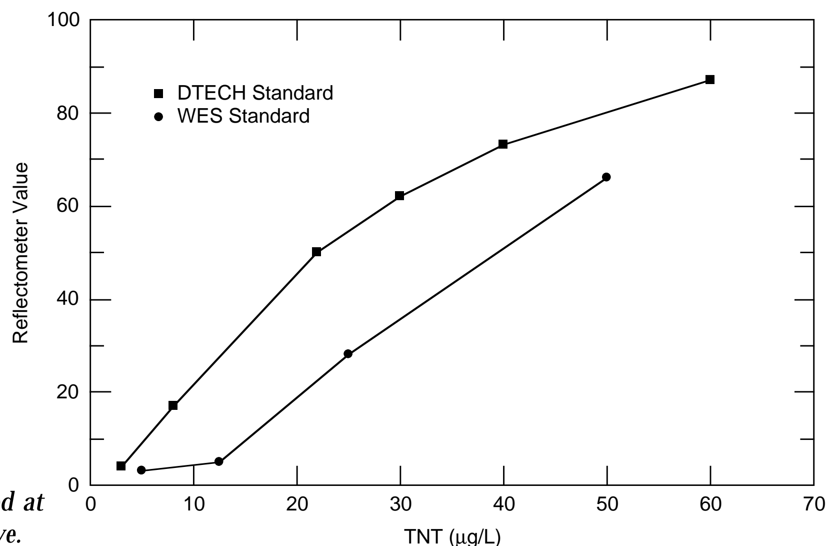


Figure 13. Standard curve produced at WES vs. the DTECH published curve.

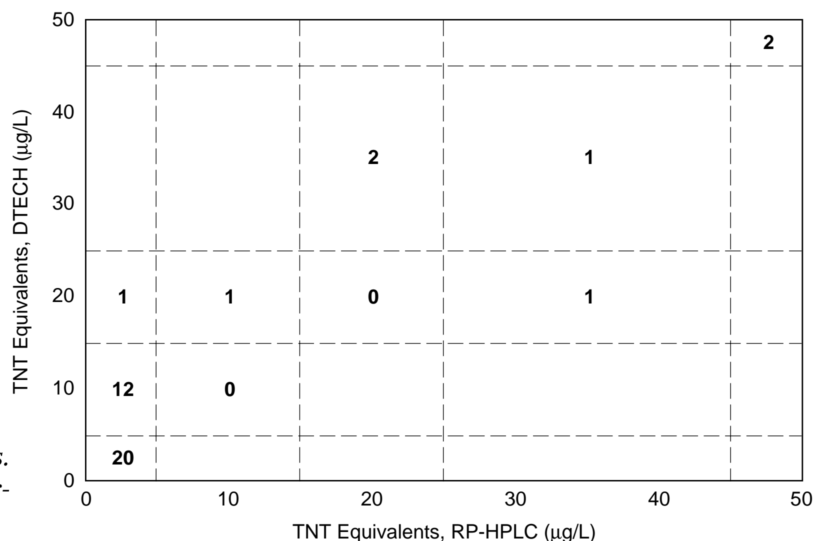


Figure 14. RP-HPLC values vs. DTECH values. Frequency of occurrence within each range.

the extreme high values for wells 731 and 743 (Table 3). The RPD criterion had to be redefined; it was acceptable if the kit value and RP-HPLC value fell within the same range. These kits failed that test over half the time. The TNT kit produced the highest number of false positives—10 out of 33 wells. The question of false negatives can be answered in two ways. The detection limit claimed by the kit is 5 µg/L, ten times higher than the next most sensitive kit (EnviroGard). At this level no false negatives occurred. However, the lack of false negatives is offset by the fact that the detection limit of 5 µg/L is higher than the 2 µg/L health advisory value proposed by the EPA for drinking water (EPA 1989). The kit did fail to detect TNT or its cross-reactive transformation products in nine samples that had measurable quantities by RP-HPLC. These kits might be considered when the detection limit is adequate and the cost of false positives is low.

The RDX kit performed so poorly that the manufacturer was contacted for advice.* Other than a color development more rapid than anticipated, the kits passed the manufacturer's quality control standards. Attempts to create a standard curve, as was done for TNT, were unsuccessful.

SUMMARY AND CONCLUSIONS

The results of this investigation of 44 groundwater wells were disappointing. None of the test kits performed as well as advertised. The quantitative assays were neither accurate nor precise enough to replace Method 8330 RP-HPLC determinations, although they can be used adequately as screening tools. Of the two DTECH assays, the RDX test failed badly by producing eight false

* Personal communication, G.Teaney, Strategic Diagnostics, Newark, Del., 1994.

negatives and six false positives. The TNT test produced ten false positives. Both of the DTECH kits had detection limits above the EPA health advisory limits.

The poor accuracy may be due to nonspecific matrix effects as indicated by the differences between performance of within-range and average values. It is also possible that the cross reactivities of the transformation products, which were identified in these samples by RP-HPLC, are different from the values quoted by the manufacturers. There could be chemicals in the wells that cause an antibody response that were not tested by the manufacturers and do not appear in the Method 8330 analysis. The poor precision may have been caused by heterogeneously distributed suspended or colloidal material. None of the kits requires filtration of water samples. At the time of analysis, most of the suspended material had settled; however, a few tests were done with samples that had been agitated and were quite turbid. The results from these tests were within the range of the results from the clear samples. The most precise kit, evaluated using the 2-µg/L EPA health advisory limit, was the Quantix, which had the most rigorous wash procedure. It uses a detergent solution and an aspirated plate-washer.

Improvements in the precision of the Ohmicron kit might be obtained by vortexing the antibody particles in the supplied detergent solution rather than merely rinsing the cluster that formed on the test tube walls next to the magnetic separator. Additional time would be required to redeposit the vortexed particles after each rinse. The precision of the EnviroGard kit might be improved by substituting a detergent solution for the recommended tap-water rinse. Improved washing steps might also improve the accuracy if positive interferences were nonspecific.

FURTHER TESTS AND CURRENT STATUS

As this report was going to press, results from a second evaluation became available (Craig et al. 1996). Well water from Umatilla Army Depot Activity, Hermiston, Oregon, and well water and soil-washing leachate from the U.S. Naval Submarine Base, Bangor, Washington, were tested using the DTECH (TNT and RDX) and Ohmicron (TNT) immunoassays, prototype antibody-based biosensors (TNT and RDX) from the Naval Research Laboratory (NRL), and EnSys colorimetric kits (TNT and RDX). Problems encountered with the DTECH RDX kit were not repeated here. DTECH supplied improved operating methods for their reflectometer that quantified the color-development time. In addition, the development time was closer to the expected 5 minutes. The results of this comparison showed that the colorimetric methods had the greatest accuracy, followed by the NRL biosensors, then the immunoassays. The accuracy of the immunoassays and biosensors were better at Umatilla than at Bangor. The high organic content and turbidity of the Bangor waters probably contributed nonspecific interferences. When contaminant levels were higher than the test ranges and the samples required dilution with distilled water, the accuracy of the immunoassays and biosensors was better. The colorimetric method involves a solid-phase extraction step, minimizing the contribution of the sample matrix.

In addition, the commercial immunoassay market made a major transition. The Quantix company has been bought out twice and the kit we used is no longer available. Ohmicron, EnviroGard, EnSys, and DTECH were combined as Strategic Diagnostics, Inc. At this time, all of their kits are available; however, it is expected that only one or two products will emerge as long-term replacements for the four existing formats.

LITERATURE CITED

Craig, H., G. Ferguson, A. Markos, A. Kusterbeck, L. Shriver-Lake, T. Jenkins, and P. Thorne (1996) Field demonstration of on-site analytical methods for TNT and RDX in ground water. In *Proceedings of the HSRC/WERC Joint Conference on the Environment, Albuquerque, New Mexico, May 21–23, 1996*. Las Cruces: New Mexico State University, p. 204–219.

EPA (1988a) Health advisory for RDX. Office of Drinking Water. Washington, D.C.: U.S. Environmental Protection Agency.

EPA (1988b) Health advisory for HMX. Office of Drinking Water. Washington, D.C.: U.S. Environmental Protection Agency.

EPA (1988c) Health advisory for 1,3-dinitrobenzene. Office of Drinking Water. Washington, D.C.: U.S. Environmental Protection Agency.

EPA (1989) Trinitrotoluene health advisory. Office of Drinking Water, Washington, D.C.: U.S. Environmental Protection Agency.

EPA (1992) Dinitrotoluene health advisory. Criteria and Standards Division, Office of Drinking Water. Washington, D.C.: U.S. Environmental Protection Agency.

EPA (1994) Nitroaromatics and nitramines by high

performance liquid chromatography. SW846 Method 8330, November. Washington, D.C.: U.S. Environmental Protection Agency.

Jenkins, T.F., and M.E. Walsh (1992) Development of field screening methods for TNT, 2,4-DNT and RDX in soil. *Talanta*, **39**: 419–428.

Jenkins, T.F., P.G. Thorne, and M.E. Walsh (1994) Field screening method for TNT and RDX in groundwater. USA Cold Regions Research and Engineering Laboratory, Special Report 94-14.

Keuchel, C., L. Well, and R. Niessner (1992a) Effect of the variation of the length of the spacer in a competitive enzyme immunoassay (ELISA) for the determination of 2,4,6-trinitrotoluene (TNT). *Freseius Journal of Analytical Chemistry*, **343**: 143.

Keuchel, C., L. Well, and R. Niessner (1992b) Enzyme-linked immunosorbent assay for the determination of 2,4,6-trinitrotoluene and related nitroaromatic compounds. *Analytical Science*, **8**: 9–12.

Keuchel, C., L. Well, and R. Niessner (1992c) Development of an enzyme immunoassay for the determination of 2,4,6-trinitrotoluene—Probing the influence of humic acids. *International Society for Optical Engineering (SPIE)*, **1716**: 44–50.

Vanderlaan, M., L. Stanker, and B. Watkins (1990) Immunochemical techniques in trace residue analysis. In *Immunoassays for Trace Chemical Analysis* (M. Vanderlaan, L.H. Stanker, B.E. Watkins, and D.W. Roberts, Ed.). Symposium Series 451. Washington, D.C.: American Chemical Society, p. 2–12.

Walsh, M.E., T.F. Jenkins, P.S. Schnitker, J.W. Elwell, and M.H. Stutz (1993) Evaluation of SW846 Method 8330 for characterization of sites contaminated with residues of high explosives. USA Cold Regions Research and Engineering Laboratory, CRREL Report 93-5.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestion for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1997	

4. TITLE AND SUBTITLE
Evaluation of Commercial Enzyme Immunoassays for the Field Screening of TNT and RDX in Water

5. FUNDING NUMBERS

6. AUTHORS
Philip G. Thorne and Karen F. Myers

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
U.S. Army Cold Regions Research and Engineering Laboratory
72 Lyme Road
Hanover, New Hampshire 03755-1290

8. PERFORMING ORGANIZATION REPORT NUMBER
Special Report 97-32

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Environmental Center
Aberdeen Proving Ground, Maryland

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
SFIM-AEC-ET-CR-97057

11. SUPPLEMENTARY NOTES For conversion of SI units to non-SI units of measurement consult *Standard Practice for Use of the International System of Units (SI)*, ASTM Standard E380-93, published by the American Society for Testing and Materials, 1916 Race St., Philadelphia, Pa. 19103.

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.
Available from NTIS, Springfield, Virginia 22161.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

Water samples from 44 monitoring wells at three military installations were analyzed for the high explosives TNT and RDX using immunoassay test kits. The accuracy and precision of the kit determinations were compared with results obtained using the RP-HPLC, EPA Method 8330. Most of the kits achieved a ±50% relative percent difference criterion over 85% of the time. One of the kits failed this test over half the time. Careful consideration must be given to interferences that may be present and unique for each application.

14. SUBJECT TERMS

Chemical analysis	Groundwater	RDX
Environmental impact	Military research	Water pollution
Explosives	TNT	

15. NUMBER OF PAGES
20

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL