Detection of Chemical Threat Agents in Drinking Water by an Early Warning Real-Time Biomonitor

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Received 13 May 2003; revised 4 August 2003; accepted 13 August 2003

ABSTRACT: Having a safe water supply for civilian organizations and military personnel is an important objective to avoid toxic contamination of civilians and soldiers. Chemical warfare (CW) agents, especially organophosphorous nerve compounds, are the most toxic of known chemical agents. The *Daphnia* Toximeter system is a continuously working test system that uses *Daphnia magna* as a sensitive organism for monitoring drinking water. Both small doses (allowable for short-term water ingestion) and graduated higher concentrations induced toxic reactions in the *Daphnia* Toximeter system, leading to alarms sounding. The system is sensitive to a wide range of CW agents and their hydrolysis products. Concentrations below acute human toxicity can be discovered in a very short time, with the actual time depending on the concentrations applied. In every case alarms were triggered within 2 h at concentrations in water low enough for that water to be allowed for use as drinking water in exceptional conditions. © 2003 Wiley Periodicals, Inc. Environ Toxicol 18: 368–374, 2003.

Keywords: chemical warfare agents; cw agents; sarin; tabun; soman; cyclosarin; cyanide, *Daphnia; Daphnia* Toximeter; drinking water surveillance

INTRODUCTION

The nature of global conflicts is changing. Recent events have increased concern about ensuring the safety of water supplies to civilian organizations and military personnel. Threats may arise from diverse directions and could confront government, municipal, military, and vital commercial systems. Toxic contamination of public water utilities with biological or chemical warfare agents can be an act of terror or of vandalism.

The toxic potency of most known chemical warfare agents is affected by hydrolysis. However, the time periods required for chemical lysis and toxicity reduction are very different, depending on temperature, accompanying chemicals, and ionic reactions.

Processing ground- or river water to make it of a quality suitable to be tap water requires an effective round-the-clock early warning alert for biological and chemical hazards.

Production of drinking water is a central task of community water suppliers and is a critical supplied to be procured and maintained by those organizing the logistics of armed forces medical services. Therefore, government organizations make significant investments in research about

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DOI 10.1002/tox.10138

both the processing of drinking water from surface water or groundwater and the assuring of a water supply for their troops. The fundamentals of our treatments in this study were guided by the U.S. Army's *Guidelines for Chemical Warfare Agents in Military Field Drinking Water* (1995).

Experiences from the biomonitoring of surface water can be extended to the biomonitoring of drinking water. As an example, during the Olympic Games of 2002 in Salt Lake City, Utah, *Daphnia* Toximeters (bbe-Moldaenke) were installed to protect the six most important waterworks in the Salt Lake valley area. A recent event demonstrated the importance of implementing early warning systems to indicate contamination and to predict life-threatening attacks. In February 2002 an attack in Rome was prevented at the last minute. The Italian police arrested four Moroccans for allegedly plotting a chemical attack in the region around the U.S. Embassy in Rome. Roughly 9 pounds of a cyanide compound as well as a map with details of water pipes that serve the embassy were found. The authorities thwarted the dangerous terrorist assault. Potassium cyanide (KCN) is easy to get as well as easy to produce; for instance, it is often used in the gold and plastic industries.

There are many chemical systems that identify chemicals by mass spectrometry or chromatography. Such systems often have significant preparation time. This lengthy preparation time and the many different chemicals required result in high costs. True real-time toxic reactions can be detected more easily by a living organism. Therefore, our goal was to prove or develop a rapid and permanent working biosystem for the detection of toxic material that would use naturally occurring biosensors, making the system practical and cost effective.

A test system should provide early and rapid information about toxic substances in water. With this objective in mind, we examined an automatically working *Daphnia* Toximeter for detecting chemical warfare agents (CWA) during drinking water processing.

MATERIAL AND METHODS

Chemicals and Methods

Sarin, tabun, cyclosarin, and soman were used in small amounts for protection research and the decontamination tests.

Sarin is very miscible with water, whereas tabun, soman, and cyclosarin are less water soluble. In wet and humid weather conditions sarin degrades swiftly. Hydrolysis is fast in an alkaline medium.

Sarin (isopropyl methylphosphonofluoridate) was dissolved in water without any solvent in doses of 100, 50, 25, and 10 μg/L of water. Each solution was made just before starting the test with daphnia. Gas chromatographic analysis of the stock solution showed a purity of 97%.

Tabun (dimethylphosporamido-cyanidate) is an organophosphorous nerve agent and is less water soluble than sarin. Its purity, determined by gas chromatography (GC), was 96%. Its solubility in water is 9.8% (25°C), and it is easy to dissolve in organic solvents (U.S. Army, 1990). Dissolved in 100 μL of ethanol (ETOH) was 106 μg of tabun (GA). From this stock solution (GA–ETOH), three doses (141, 71, and 35.5 μg/L of water) were applied to daphnia in three separate tests.

Soman (pinacolyl-methyl-phosphonofluoridate) is miscible in polar and nonpolar solvents and is 2.1% soluble in water at 20°C and 20% soluble at 25°C. Purity of 70% was determined by flame atomic emission spectrometry (Perkin–Elmer). Into 45 μL of ETOH was dissolved 32 μg of soman (GD), and then three doses (64, 32, and 6.4 μg GD–ETOH/L of water) were prepared.

Cyclosarin (cyclohexyl-methyl-phosphonofluoridate) has a structure similar to sarin, except with a cyclohexyl group instead of a 2-propyl group. Its solubility in water is 0.37% at 20°C (U.S. Army, 1990). Purity of 98% was established. It is very stable and only hydrolyzes when heated or with alkalis. In 50 μL of ETOH was dissolved 120 μg of cyclosarin (GF). A concentration of 100 μL GF–ETOH/L of water is equivalent to 240 μg GF/L. Doses of 240, 120, and 60 μg/L were used.

Potassium cyanide was dissolved in water in concentrations of 1, 2, 5, and 10 mg/L of water.

The maximum allowable concentrations of CW agents in drinking water are laid down in Appendix B of the *Guidelines for Chemical Warfare Agents in Military Field Drinking Water Standards* (U.S. Army, 1995).

Tap water of drinking quality from a German community, produced according to the official regulations of the German Drinking Water Decree 2001 and with no detectable free chlorine and 6.8 mg of chloride, was used as a negative control. In addition, 200 mg NaCl/L of water was added to the tap water to test reactions of *Daphnia*. This was done because a threshold value of 250 mg of NaCl is allowed in drinking water (German/European Drinking Water Regulation, 2001). Other compounds are allowed only in technologically unavoidable traces. Another control test was performed using tap water flowing through a copper water pipe as a control. Positive controls were sodium hypochlorite, added to produce free chlorine at a maximum concentration of 0.32 mg/L of water, and ethyl alcohol at a concentration of 1 mL/L of water.

The decontamination procedure of the *Daphnia* Toximeter was performed with potassium hydroxide (KOH), and the entire circuit system was rinsed with bidistilled water after that.

For the gradation of different doses in the experiments, principally data from Appendix I of the USAMRIID *Medical Management Handbook* (2001) were used. Purity of the CW agents was determined by gas chromatography (GC) using a defined standard.

TABLE I. A camera monitor allows a macroscopic view and recording traces of Daphnia movements for identification of number and behavior of Daphnia magna. The table describes the kind of observed behavior. The last column expresses the exclusiveness of the behavioral class for indicating the appearance of toxic substances. If more than 9 toxicity points are added at about the same time an alarm will be released

Parameter	Description	Toxicity Points
Velocity	The average swimming speed of Daphnia was calculated under normal conditions. Deviation of speed will increase or decrease with toxic reactions.	5
Fractal dimension	This parameter evaluates sudden changes in the movement of Daphnia. Swimming along a straight line has the mathematical dimension 1. Deviation from this straight line alters the dimension. In particular Daphnia become hectic and turn themselves around in small circles if there is a contact with toxic substances.	3
V-class-index	The average speeds can be determined into classes. The percentage of Daphnia swimming faster or slower than 0.2 cm/s (medium speed) alters the class-index. The classes are added together and transmitted into a measurement.	5
Height	An obvious toxic event mostly induces the average swimming height to drop down suddenly.	2
Distance	Group behavior of Daphnia is assessed in this parameter by evaluating changes in distance between each Daphnias.	3
Number	Death of Daphnia indicates a contamination of water. Deviation in number between the current and the initial number of Daphnia is a very important parameter.	up to 20

Organisms and Test System

As a sensitive test organism *Daphnia magna* Straus ages 5–7 days were used. This strain originates from the German Federal Environmental Agency (Berlin, Germany) and was bred continuously by the German Armed Forces Institute for Protection Technologies. The toximeter system has two devices: one part consists of a glass chamber for the organisms, a camera for observing the swimming behavior of daphnia, and a tube-pump system; the second part consists of a computer and monitor. The daphnia chamber was flowed through by a sample circuit stream. To keep the *Daphnia* from escaping, a metal grid was installed. Temperature regulation was provided by an internal Peltier element. The digital camera was focused on the chamber with the *Daphnia,* which was connected to an online computer system that stored data as *Daphnia* speed (cm/s), *Daphnia* swimming height (cm), distances between *Daphnia* (cm), fractal dimension (geometrical course of a curve), velocity class index, and number of active *Daphnia* (Lechelt et al., 2000). Before starting each test, there was a 6-h adaptation period for the daphnia. Statistical changes (computed using the Hinkley calculation, a mathematical tool, and the slope gradient) in each of these behavioral classes contribute to the so-called toxicity index (Lechelt et al., 2000). The parameters are described in Table I. Their quality and their contribution to the toxicity index are expressed in the weighting added in the last column of the table. When the toximeter system is running, in cases in which a statistical deviation has occurred within the last 4.5 h, each behavioral class would be added to the designated number of toxicity points and to the toxicity index. If the index exceeds 9 points, an alarm occurs. This method of evaluation increases sensitivity and reduces false alarms (Lechelt et al., 2000). The sensitivity of the toxicity index in inducing amber or red alarms can be altered based on practical experience.

RESULTS

It is important to get early information about the toxic burden of raw water during or prior to processing it into drinking water. In general, detection of a toxic reaction is the result of two parameters: (1) a chemical or biological compound present, and (2) a sensitive organism. *Daphnia magna* is a water insect that is well known for environmental water surveillance (ISO/DIS 6341, 1994). Its behavior and swimming conditions under both nontoxic and toxic burdens are well defined. A deviating swimming behavior could be the result of avoiding a substance in the water or of impairment of metabolic reactions after toxic influences. As an example, Figure 1 shows the drastic changes in some behavioral classes of *Daphnia* movements after treatment with sarin at a concentration of 10 μg/L.

To avoid false-positive reactions, the behavior of daphnia were tested with both 6.8 and 200 mg NaCl/L of tap water. No abnormal reactions to both NaCl content and addition of ethanol only were found over a test period of 24 h. By contrast, drinking water flowing through a copper pipe induced toxic reactions in daphnia organisms. Content of copper ions in the drinking water (pH 8.3) was determined by atomic absorption spectrometry and reached values of 294.7–323.2 μg/L of water. Fifty percent of the daphnia died within 4 h, and all were dead within 5.75 h. After chlorination of raw water and disinfection with sodium hypochlorite, the threshold chlorine concentrations

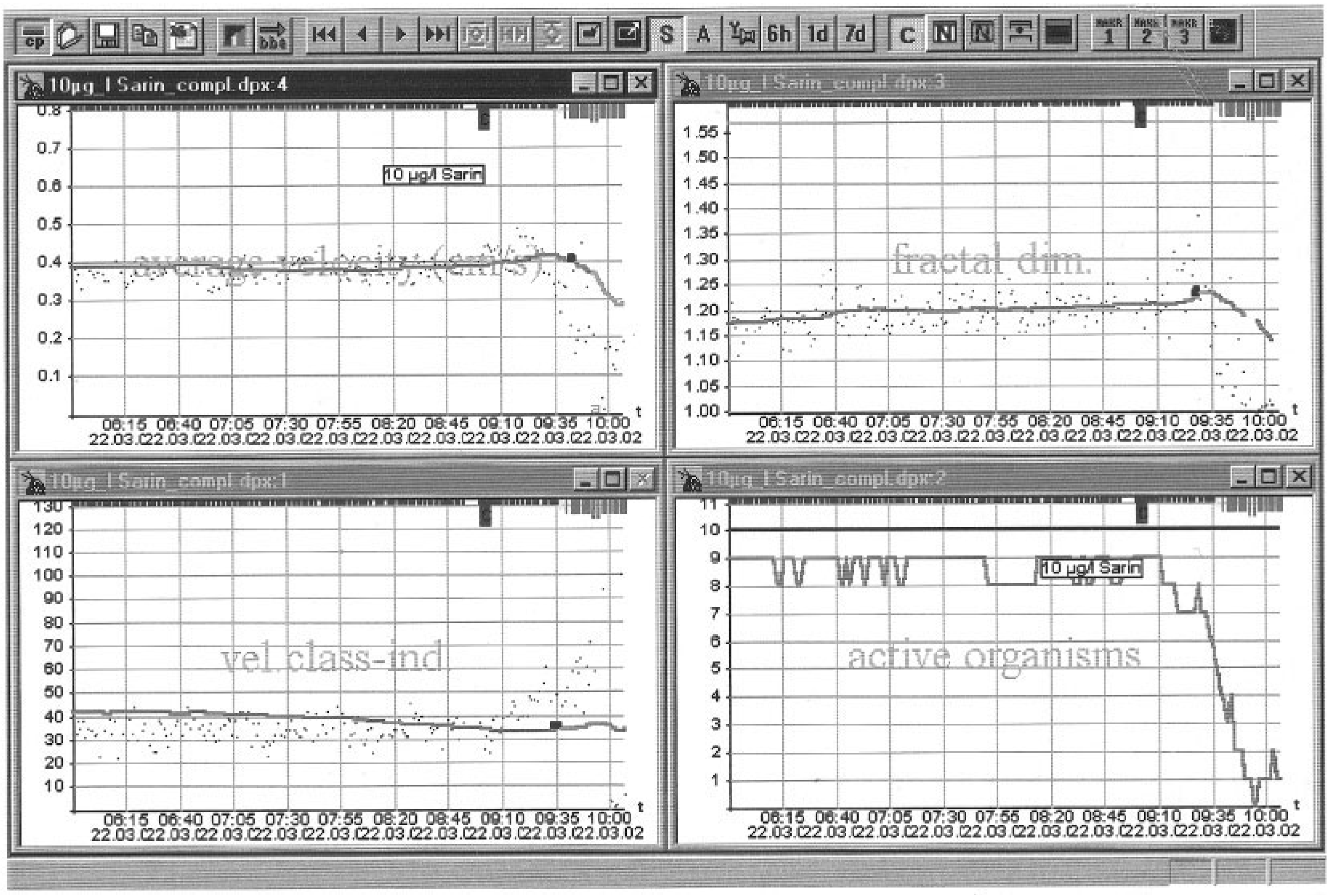

Fig. 1. Evaluation of treatment with sarin at concentration of 10 µg/L: top left, average velocity; top right, fractal dimension; lower left, velocity class index; lower right, active organisms. Delays of the current approach because of tubing, etc., have been considered. There is one data point for each minute, with evaluation taking place every 2 min. The toxicity points will now be added to the toxicity index. [C: start of induction of chemical; bar (upper right): alarm 25 min after inducing toxin in the sample water; solid line: predicted values, accounted for by linear regression from data of the previous 4.5 h of each data point; rectangle: statistically significant difference between predicted and measured values.] As can be seen from the tracks of the movement (frac. dimension, top right), very soon after starting the treatment cycle, movement of daphnids increased. Statistical analysis showed that behavior deviating for more than 15 min would be unusual, with the percentage of slow and fast movements thereafter considered abnormal. Recognition of an additional deviation in the parameter median speed led to an announced alarm.

can reach a maximum of 0.3 mg free chlorine/L and 0.6 mg/L of drinking water, respectively. *Daphnia* were tested with 0.32 mg chlorine/L of water. Within 40 min the number of living *Daphnia* was significantly reduced, and after 73 min all were dead.

Sarin (GB)was the first warfare agent tested, as it is the most water-miscible CW substance. The LD_{50} dose used in laboratory mice is 100 µg sarin/kg of body weight, which was the basis for our tests. Progressively lower doses were tested to get a dose–response curve. The time to total loss of the organisms began at 4 min and went up to 64 min as the doses were decreased. The so-called permissible value for short-term ingestion of sarin in military field drinking water by soldiers is 10 µg sarin/L of water (U.S. Army, 1995). Applying this small dose, the first signs of reduced motility in *Daphnia* were seen after 2 h and 100% immobilization after 6 h. An alarm activated by exceeding the toxicity index went off after 150 min.

Soman, tabun, and cyclosarin are less water soluble but nevertheless are toxic in water under special conditions, as shown in Table II. The small soman dose of 6.4 µg/L of water is within the dose range interpreted as an allowable concentration for short-term (7 days and 5 L/day) water ingestion for personnel under military field conditions. This

TABLE II. Toxic effects of CW agents in a Daphnia-Toximeter-System

Substance	Dose: μg/l Water	Increasing Motion Indicating a Toxic Reaction: Time Interval:	50% Inactivated Time Interval:	100% Inactivated Time Interval:
Sarin (GB)	100		2 min	4 min
	50		3 min	8 min
	25		27 min	64 min
	10	25 min	120 min	360 min
Soman (GD)	64	22 min	38 min	47 min
	32	69 min	82 min	114 min
	6, 4	80 min	270 min	330 min
Tabun (GA)	141	18 min	24 min	31 min
	71	45 min	66 min	85 min
	35, 5	55 min	65 min	99 min
Cyclosarin (GF)	240	15 min	20 min	25 min
	120	16 min	20 min	26 min
	60	34 min	52 min	65 min

concentration led to a first alarm after 270 min in the *Daphnia* Toximeter, the point at which 50% of the *Daphnia* were inactivated.

At higher doses (141, 71, and 35.5 μg/L), tabun showed a faster and more significant intoxication than did soman (Table II). The solubility of cyclosarin in water is limited to 2 g/100 mL. The alarm for 50% inactivated organisms was triggered 20–52 min after exposure to the tested doses (240, 120, and 60 μg/L).

Table III shows the toxic effects on daphnids when potassium cyanide (KCN) is dissolved in drinking water. A dose of 10 mg/L of water is at the upper range of allowable water intake for a 70-kg person in a 7-day period according to guidelines for field drinking water conditions (U.S. Guidelines, 1995), whereas 5 and 2 mg/L are below the defined threshold.

DISCUSSION

Nerve agents are the most toxic to human beings of known chemical agents. They are health hazards in their liquid and vapor states and in high doses can cause death within minutes of exposure. These toxic agents are considered major threat agents, not only in armed conflicts but also in terrorist attacks.

Water contaminated with CW agents may be a toxic hazard when it is used for drinking, for washing, and in food preparation. Although many CW agents hydrolyze in water, this is not a satisfactory method of decontamination because the efficiency of hydrolysis depends on pH, temperature, and water ingredients. In addition, the products of hydrolysis also might be toxic. Degradation processes include hydrolysis, oxidation, microbial degradation, and photolysis and some major degradation products may be significantly persistent (Munro et al., 1999).

Having early warning systems for drinking water processes continues to increase in importance, and the *Daphnia* Toximeter is a permanent, continuously working instrument used in these investigations. It is a flow-through water system using living organisms (*Daphnia magna*) that react when toxic substances are in the water. The surfaces, digestion tracts, and gill systems of these organisms come in contact with a toxic substance if dissolved or suspended in water. In addition, most insects decompose alkylphosphates much more slowly than mammals. Therefore, this surveillance system is very sensitive and usable as an indicator of a sum of toxic parameters.

This toximeter system was used during the Winter Olympic Games in Salt Lake City to avoid having toxic substances in the drinking water process. Raw water was taken

TABLE III. Evaluation of toxic effects of KCN

Substance	Dose: mg/l Water	Increasing Motion Indicating a Toxic Reaction: Time Interval:	50% Inactivated Time Interval:	100% Inactivated Time Interval:
KCN	10	25 min	60 min	—
	5	30 min	70 min	—
	2	90 min	—	—
	1	190 min	—	—

from rivers filled with melting ice and snow from the mountains. Whereas many human toxic compounds including some pesticides were tested, experience with testing on warfare agents was absent.

A tool for the surveillance of drinking water under military field conditions is a biochemical method to indicate the inhibition of the enzyme acetylcholinesterase (Strömmer et al., 2002), when organophosphorous agents are added. Cyanides were detected by adding isonicotinic acid and 1,4-dimethylbarbituric acid into a test reaction. The existence of these toxic agents was indicated by a color reaction. However, this test system is not qualified for permanent surveillance, as different toxic substances must be identified by different chemical reactions.

Tap water with an allowable level of the ingredient NaCl of up to 210 mg did not induce an alarm. However, tap water with a pH of 8.3 flowing through a copper pipe was toxic for daphnia and triggered an alarm in the daphnia system after 4 h, 40 min. Beyond that, the disinfection of drinking water in processing by use of chlorine is a procedure commonly used in many states. However, chlorine is toxic for many marine organisms (Brooks et al., 1978) and can produce trihalomethanes, which are toxic, too. Therefore, a threshold value of 0.01 mg trihalomethane/L should not be exceeded. In our experiments even small amounts of chlorine (0.3 mg chlorine/L of water) induced a significant toxic reaction to daphnia after 40 min. Such chlorination can be a further step in the decomposition of warfare agents after hydrolysis and can help to prevent poisoning.

Sarin (GB) soluted in very small amounts (10 μg/L) induced a warning signal in the *Daphnia* Toximeter system. This concentration is under the detection limit (20 μg/L) of a test kit (M272) of the U.S. Army (1983, 1986). Also, a dose of 10 μg of sarin/L will release an alarm within 15–25 min. After 27 min 50% of the *Daphnia* were inactivated, and in 64 min all the *Daphnia* were dead. By doubling the doses to 50 and 100 μg/L, significant inactivation occurred within 2–8 min. These short time reactions indicated it is possible to stop a public water supply system of in order to prevent people from ingesting contaminated water.

The solubility of tabun (GA) in water is 9.8% at 25°C (Sidell, 1995). Toxicology data in the literature on the LD_{50} of GA show it varying between 1000 and 4000 mg/70 kg of human body weight percutaneously. In our considerations we preferred the LD_{50} of 1000 mg/70 kg and from that deduced the doses of 141, 71, and 35.5 μg/L of water. A 50% level of inactivated Daphnia was found in time intervals of 24–65 min, and total inactivation (100%) occurred after 31–99 min in relation to the doses used. This shows that it is very difficult to compare the *Daphnia* reaction times between CW agents dissolved in water on the basis of LD_{50} values estimated for human beings if the method of application differs, as the agents can be applied by inhalation, percutaneous application, and subcutaneous injection methods.

Soman is more toxic on skin than are sarin and tabun. Its LD_{50}, 64 μg/kg of body weight in laboratory mice, was given in Appendix I of the *Handbook of the USAMRIID* (2001). This was the initial dose used in the water treatment with Daphnia, followed by doses that were 50% (half) and 10% of that dose, comparable to the allowed concentration for short-term water ingestion under military field conditions. Increasing motion of the *Daphnia* was seen after 22 min, and 16 min later 50% of the *Daphnia* were inactivated after a dose of 64 μg/L. On the basis of the same LD_{50}, both time intervals for soman were longer than the tabun (141 μg/L) intervals (18 and 24 min). The reaction time of 50% inactivated *Daphnia* is short enough to stop a water supplier system before tap water reaches most consumers. Very small doses (6.4 μg/L) of soman, three times smaller than the allowable concentration of man under field conditions of organophosphorus nerve agents, induced toxic reactions (50% inactivated) within 2.5 h in *Daphnia*.

Toxicological data about cyclosarin (GF) in the literature are sparse. Van Beest et al. (1995) calculated a liquid LD_{50} dose of 850 mg/human, whereas the U.S. Army Medical Research Institute (2001) indicated a dose of 30 mg on skin.

Subcutaneous application of cyclosarin to mice (Clement, 1991, 1994) led to an LD_{50} of 243 μg/kg of body weight. This concentration was the starting point for the *Daphnia* investigations because the basis of sarin investigation also was provided by mice data. Cyclosarin seemed to be less toxic than sarin in water. A dose of 240 μg/L led to 50% of organisms inactivated in 20 min. However, half the dose (120 μg/L) induced the same lethal reaction time, 20 min, and the time interval until 100% inactivation of *Daphnia* was found to be nearly the same time (25 and 26 min) in both doses. This led to the conclusion that GF had a longer metabolic conversion in the body, ending in sudden lethal effects. A dose of 60 μg/L prolonged the time interval to reach 50% inactivated organisms to 52 min.

Potassium cyanide is not as dangerous as organophosphorous agents. Human lethality (LC_{50} at a body weight of 70 kg) is induced at 180 mg/L (Franke, 1977). Cyanides act quickly and are quickly distributed throughout the body. This substance inhibits normal respiration in the cells so that oxygen cannot be used because cyanide binds strongly to red blood cells. However, its concentration in human tissues decreases rapidly after a single dose. The *Daphnia* Toximeter releases an alarm at concentrations of about 5 mg/L within 30 min. In most cases this capability would be sufficient for an effective alarm system.

The *Daphnia* Toximeter is a permanent working device for toxicological surveillance of drinking water against assaults with CW nerve agents and cyanides. This system is designed to identify not only CW agents but to give alarms if any toxic substances are in a water system. And it also reacts if very small amounts of CW substances are dissolved that are below an allowable drinking water threshold value.

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