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Note

A simplified dehydrogenase enzyme assay in contaminated sediment using 2-(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride

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Abstract

2-(*p*-Iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) accepts electrons from dehydrogenase enzymes and is reduced to a red-colored formazan (INTF), which can be quantified by colorimetric analysis. Use of previously published methods for this technique was unsuccessful due to background chemical reactions from high levels of polycyclic aromatic hydrocarbons (PAHs) and metals in the sediments. A modified method using acetonitrile extraction of the INTF was efficient and did not chemically reduce INT. This activity method is simple, quick, inexpensive and precise.

Keywords: 2-(*p*-Iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride; Dehydrogenase enzyme; Polycyclic aromatic hydrocarbon

To examine the potential for bioremediation of industrially contaminated sediments in the Lower Mahoning River of northeast Ohio, USA, microbial activity measurements were made by estimating dehydrogenase activity using 2-(*p*-Iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Due to the contamination of these primarily anaerobic sediments [metals: 204 g/kg, polycyclic aromatic hydrocarbons (PAHs): 32 mg/kg], previous methods (Trevors, 1983; Camina et al., 1997; Mathew and Obbard, 2001; von Mersi and Schinner, 1991) were unsuccessful as many of the previously reported solvents [i.e. *N*,*N*-dimethylformamide (*N*,*N*-DMF), acetone and dichloromethane] caused chemical reduction of INT in sterilized sediments.

Microbes in sediments are usually found adhered to sediment particles, which create difficulty in quantitating them using conventional microbial methods (Lutz-Arend, 1994; Richards, 1999; Riis et al., 1998). The reduction of tetrazolium salts has been used as an indicator for dehydrogenase activity by a wide range of microbes from bacteria to fungi and algae (Curl and Sandberg, 1961; Fonseca et al., 2001, Maurines-Carboneill et al., 1998; Smith and McFeters, 1997; Trevors, 1982a,b; Trevors et al., 1983a,b). Estimating microbial activity by measuring INT reduction was the method of choice as it could be performed in short incubations, it does not require the use of radioactive

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substrates and only viable cells are measured (Trevors et al., 1983a).

Dehydrogenases are oxidoreductase enzymes that take part in respiration in microbial cells. These enzymes oxidize organic compounds by the transfer of electron pairs from a substrate to nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$) forming NADH or NADPH, respectively (Smith and McFeters, 1997). This is a vital part of the electron transport system of a cell (Crane et al., 1991). The use of a tetrazolium salt is a widely accepted method for measuring redox reactions in cells (Smith and McFeters, 1997). INT successfully competes with NAD^+ and $NADP^+$ for electrons. INT inserts between ubiquinone and cytochrome *b* in the electron transport chain (Maurines-Carboneill et al., 1998). As INT accepts electrons, it is reduced to a red formazan (Altmann, 1969; Curl and Sandberg, 1961). Colorimetric methods based on INT reduction provide an accurate assay of dehydrogenase activities under both anaerobic and aerobic conditions (Bhupathiraju et al., 1999; Trevors et al., 1982; von Mersi and Schinner, 1991).

5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) and triphenyltetrazolium chloride (TTC) have also been used to measure dehydrogenase activity in environmental samples (Bhupathiraju et al., 1999; Lopez-Amoros et al., 1998; Maurines-Carboneill et al., 1998; Wuertz et al., 1998). INT and TTC have been shown to perform well in anaerobic systems, while CTC has not been established to work anaerobically (Bhupathiraju et al., 1999). TTC reduction is inhibited by oxygen making it useful in anaerobic conditions only (Camina et al., 1997). INT was chosen for this study, because it was established to perform in both aerobic and anaerobic conditions (Trevors, 1983, 1984).

Sediment was hand collected in shallow water using plexiglass cores tubes (51 cm long, 5 cm in diameter) from the Lower Mahoning River, 33 km upstream from where the Mahoning River joins with the Shenango River to form the Beaver River. Sediment was transported to the laboratory on ice and was processed within 2 h of collection. The core was extruded in a glove bag under a nitrogen atmosphere. Sediment from the upper 5–10 cm of the core was discarded. Sediment from the next 10 cm was homogenized and 0.10-g samples were weighed into scintillation vials, in triplicate and water (0.5 ml of 18 MΩ cm^{-1}) was added. All activity measurements were performed in the dark and/or dark room conditions, as the tetrazolium compound is light sensitive. This method was performed anaerobically in a glove bag under nitrogen flow, as well as aerobically under ambient conditions. INT solution was prepared by adding 100-μl *N*,*N*-DMF to 0.03 g INT and stirring with a glass rod. This was brought to volume in a 50-ml volumetric flask with water and sonicated with gentle heating (von Mersi and Schinner, 1991).

The samples for metabolic measurements were vortexed and incubated at room temperature for 30 min. Next, 0.5 ml of 1.08 mM INT solution in water was added and mixed; these were incubated for various times at room temperature. Following incubation, metabolic activity was stopped by adding 3.0 ml of acetonitrile and INT formazan was extracted at room temperature for 10 min. The samples were vacuum filtered through Whatman No. 40 filter paper and the sediment washed with 10.0 ml of acetonitrile. The absorbance of the combined filtrate was determined with a Shimadzu UV-260 spectrophotometer at 490 nm.

Control samples were killed with 3.0 ml acetonitrile before addition of INT to prevent reduction. Then, 0.5 ml of 1.08 mM INT was added and allowed to extract for 10 min before filtering.

Sample INTF concentrations were determined from the linear least squares best-fit line from a standard curve of INTF solutions in acetonitrile. Total activity (μmol INTF/g dry sediment) was determined by correcting the sample INTF concentration for sediment moisture content. Microbial activities were calculated by subtracting the activity found in the respective control samples.

Efficacy of acetonitrile to extract the formazan from the sediments was determined. No non-microbial reduction of INT was detected (Table 1). *N*,*N*-DMF, acetone, ethyl acetate and 10% formalin caused chemical reduction of INT in the presence of autoclaved sediments. Alcohols did not cause chemical reduction, but required large volumes for reproducible INTF extraction.

Sixty-minute incubations with different INT concentrations (data not shown) showed that 0.54 mM gave the lowest variance and yielded absorbencies in the linear range of the INTF standard curve (0–53

Table 1
Results from solvent trials for microbial activity by measuring INT reduced to INTF

Solvent	Artificial reduction by solvent	Volume of solvent required for extraction (ml)
Ethanol	No	35
Methanol	No	40
N,*N*-DMF	Yes	Not applicable
1:1 *N*,*N*-DMF/ethanol	Yes	Not applicable
Acetone	Yes	Not applicable
Acetonitrile	No	10
Isopropanol	No	37
Ethyl acetate	Yes	Not applicable
10% Formalin	Yes	Not applicable

μmol/l). Previously published methods used INT concentrations of 9.88 mM, which were then diluted for analysis (Camina et al., 1997; Mathew and Obbard, 2001; von Mersi and Schinner, 1991). This present method uses lower INT concentrations, thus eliminating variance introduced by additional dilutions.

Results from time-course incubations (Fig. 1) indicated that 2- and 60-min incubations can be used to estimate the initial rate and the final extent of INT reduction, respectively. Microbial biomass findings (data not shown) indicated that the method was not

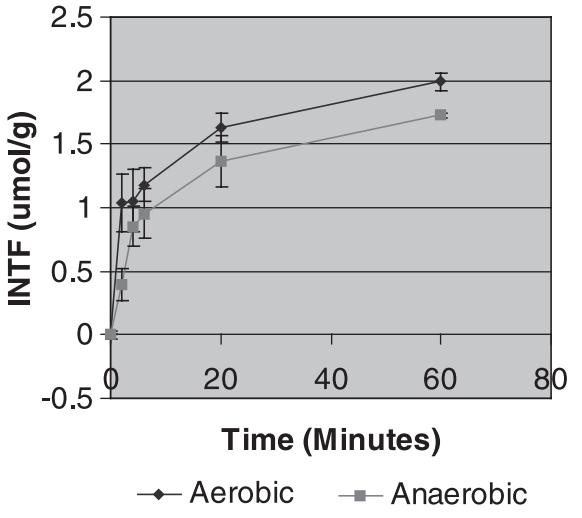

Fig. 1. The effect of incubation time for INT reduction aerobically vs. anaerobically in sediments from the Lower Mahoning River.

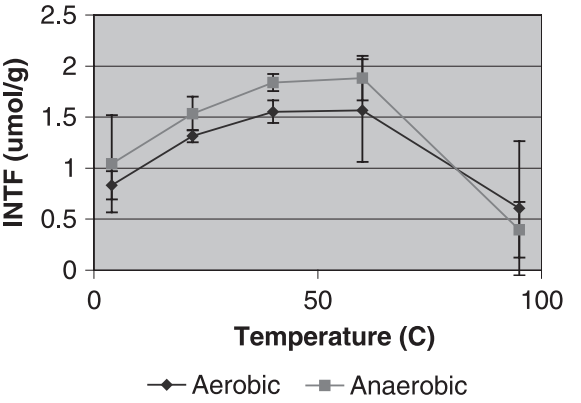

Fig. 2. Reduction from INT to INTF at different temperatures in sediments of the Lower Mahoning River (60-min incubations). Abiotic controls using acetonitrile were subtracted from metabolic samples.

just an indirect method for measuring biomass, as there was no direct correlation between biomass and activity.

Incubations at various temperatures showed an increase of INT reduction as the temperatures increased up to 60 °C (Fig. 2). Incubation at 95 °C inhibited INT reduction, indicating that INTF formation was biologically mediated at the lower temperatures.

Acetonitrile was found to be the most effective and efficient abiotic control. Twice autoclaved and aceto-

Table 2
Comparison of microbial activity results to previously published studies

Substrate	INTF (μmol/g/h)
Lower Mahoning River sediment[a]	0.8–3.2
Sandstone from Portchester Castle, UK[b]	0.002–0.008
Beach sediment contaminated with crude oil (Singapore)[c]	0.005–0.02
Arable soil (Austria)[d]	0.2–0.6
Sandy loam from Cambridge Research Station (Ontario, Canada)[e]	0.04–0.2

[a] This study.
[b] Taylor and May (2000).
[c] Mathew and Obbard (2001).
[d] von Mersi and Schinner (1991).
[e] Trevors et al. (1982).

nitrile-inoculated sediment controls showed little INT reduction and were near identical (data not shown). Sodium azide, typically used as an abiotic control, shuts down microbial respiration by reducing cytochrome *b*, thus terminating electron transport and dehydrogenase activities (Ning et al., 1996; Rozycki and Bartha, 1981; Wilson and Chance, 1967). However, in these sediments, INTF production occurred at levels significantly above the acetonitrile controls even following a 24-h preincubation in the presence of 20 mM sodium azide.

A comparison of microbial activity determined by INT reduction from different sites shows a wide range of reduction rates (Table 2). Microbial activity in sediments from the Lower Mahoning River is in the same order of magnitude as the highest measurements determined at other sites. Substrates used in previous studies included non-contaminated soils, sandstone and sediments (Brohon et al., 1999; Maurines-Carboneill et al., 1998; Taylor and May, 2000; Trevors, 1984; von Mersi and Schinner, 1991; Wuertz et al., 1998). A previous study optimized INT reduction in organic contaminated substrates (Mathew and Obbard, 2001). They found that *N*,*N*-DMF, tetrahydrofuran and methanol to be effective solvents for extraction. In metal-contaminated soil, INT reduction was elevated in the presence of copper. Several other metals did not effect the INTF production, nor were there any problems associated with solvent-induced chemical reduction (Obbard, 2001). None of the above studies were conducted with substrates that contained a combination of organic and inorganic (heavy metal) contaminants. Interference in established methods was likely a combination of the nature of contaminants (both inorganic and organic) and their interactions with each other and the solvents that were investigated.

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