

# 5-Cyano-2,3-ditoyl tetrazolium chloride (CTC) reduction in a mesophilic anaerobic digester: Measuring redox behavior, differentiating abiotic reduction, and comparing FISH response as an activity indicator

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## Abstract

The tetrazolium salt 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) has been widely applied to assess microbiological activity in environmental samples. CTC reduction has previously been quantified in a variety of anaerobic systems (i.e., fermentative, nitrate reducing, sulfate reducing) using direct microscopy, solvent extraction, and flow cytometry. In this work, extracellular CTC reduction was observed and distinguished from its intercellular counterparts by the amorphous character and near uniform fluorescence of the resulting formazan precipitates (CTF). Fluorescence yielded by non-cellular-associated formazan precipitates bleached much more rapidly than CTF formed within cells under identical UV exposure (<2 min). Dehydrogenase activity assays and fluorescent in situ hybridization (FISH) were simultaneously carried out in microcosms containing active anaerobic digester biomass, propylene glycol, and settled sewage centrate for direct comparison. In substrate limited microcosms, quantitative FISH measurements remained well above their detection limit indicating sustained intercellular ribosomal RNA concentrations over a 5-day period, while dehydrogenase assays (CTC) decreased to background levels within 14 h of substrate limitation. Results from this work suggest that CTC reduction in cell-free samples may impede accurate enzyme activity measurements, particularly when quantification involves solvent extraction, flow cytometry, or software-aided counting. In addition, activity assessment in anaerobic digesters using FISH and CTC reduction assays may be comparable until substrate becomes limited.

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## 1. Introduction

The ubiquitous electron transport system (ETS) present in many living cells allows indirect measure-

ment of respiratory activity in a wide range of prokaryotes and eukaryotes. The tetrazolium salt 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has gained wide application in ecological and environmental studies as compared to other tetrazolium salts (i.e., INT) because the reduced form, a fluorescent red intracellular CTC–formazan (CTF) precipitate, is easily detected with an epifluorescent microscope (Rodriguez et al., 1992) and retains its fluorescence upon storage for up to 2 days (Yu et al., 1995). The CTF produced can be quantified in whole cells by direct microscopy (Rodriguez et al., 1992; Pyle et al., 1995) and flow cytometry (Kaprelyants and Kell, 1993; Yu and McFeters, 1994; del Giorgio et al., 1997) or in cell extracts using spectrophotometry (Smith and McFeters, 1997; Bhupathiraju et al., 1999).

CTC has been used to assess microbiological activity in environmental samples spanning a broad range of oxidation/reduction conditions including wastewater, seawater, groundwater, drinking water, biofilm, sediment and soil samples (Rodriguez et al., 1992; Schaule et al., 1993; Winding et al., 1994; Yu et al., 1995; Choi et al., 1999). CTC has been used extensively for quantifying microbial activity in aerobic environments (Rodriguez et al., 1992; Kaprelyants and Kell, 1993). Kaprelyants and Kell (1993) reported that CTC was reduced directly by ETS-associated dehydrogenase enzymes in *Micrococcus luteus*, and Lopez-Amoros et al. (1995) demonstrated that CTC could be reduced by NAD(P)H in fixed (formalin) *Escherichia coli*. Smith and McFeters (1997) further clarified CTC reduction behavior in aerobic *E. coli* K-12 cultures. However, CTC application under anaerobic conditions has only recently been investigated (Smith and McFeters, 1997; Bhupathiraju et al., 1999; Proctor and Souza, 2001). CTC was reduced to formazan by a variety of anaerobes during all phases of growth (Bhupathiraju et al., 1999), indicating that intercellular enzymes (e.g., dehydrogenases) present during anaerobic processes (fermentation, nitrate reduction) are capable of reducing CTC. However, further understanding of the anaerobic processes involved in CTC reduction may be complicated, because anaerobic microorganisms utilize different ETS enzymes and pathways than their aerobic counterparts.

Some researchers have suggested and observed abiotic reduction of tetrazolium salts in environ-

mental samples (e.g., sediments and media prepared with reducing agents) where the redox potential was low (Rodriguez et al., 1992; Schaule et al., 1993; Smith and McFeters, 1997; Bhupathiraju et al., 1999). The midpoint reduction of CTC has been measured at  $-200$  mV; however, a weakly fluorescent, poorly localized intracellular formazan has been observed at higher redox potentials ( $-50$  mV) (Smith and McFeters, 1997). Bhupathiraju et al. (1999) noted abiotic reduction in the presence of ferrous iron and various reducing agents (e.g., ascorbic acid, cysteine hydrochloride, disodium sulfide) depending upon pH, concentration of reducing agent, and exposure time. Also, Smith and McFeters (1997) demonstrated reduction of CTC by membrane vesicles used to model membrane bound ETS components (in absence of viable cells) in reducing environments such as sediments. These results suggest that CTC may be used to quantify metabolic activity of anaerobic digesters as long as extracellular and matrix reduction mechanisms are considered.

Microbial activity in anaerobic environments may also be quantified by hybridizing labeled oligonucleotide probes, because with many genera, ribosomal RNA (rRNA) concentration varies in proportion to growth rate (DeLong et al., 1989; Poulsen et al., 1993). The application of comparative rRNA sequencing permits the direct study of microbial ecology in natural and engineered systems (Wagner et al., 1994; Pace et al., 1986). Single cells can be identified and quantified based on whole-cell hybridization of fluorescent dye oligonucleotide probes to rRNA (Amann et al., 1990; Raskin et al., 1994b). More recently, fluorescently or radioactive-labeled probes have been used to describe community structure in anaerobic bioreactors (Gruden et al., 2001; Merkel et al., 1999; Elferink et al., 1998; Raskin et al., 1994a,b). An estimate of active cell numbers present in anaerobic digesters has been approximated by the application of two domain-level probes: *Eubacter* (EUB338) and *Archaea* (ARC915) (Gruden et al., 2001; Raskin et al., 1994a,b; Pace, 1996; Merkel et al., 1999). We report here a comparison of two independent anaerobic activity assays in environmental samples: one based on dehydrogenase activity, and the other based on ribosomal RNA content.

## 2. Materials and methods

### 2.1. Anaerobic systems

The digesters used in this research were seeded with actively digesting sludge collected from the 17 million gallons per day (MGD) Boulder Wastewater Treatment Facility (BWWTP) in Boulder, CO, which is a trickling filter-solids contact facility with mesophilic anaerobic digesters. Laboratory digesters were stirred intermittently with Teflon coated stir bars (1.5 cm diameter  $\times$  20 cm length) and shaken vigorously by hand every 48 h to ensure adequate mixing. The digesters were operated at  $37 \pm 2$  °C and pH was maintained at  $7.0 \pm 0.2$  units. These bench-scale digesters were fed biosolids (400 ml) from primary sedimentation tanks (BWWTP) amended with 3.5 ml propylene glycol (PG) (80% of total COD, total COD = 7350 mg COD/l day) and were operated at a 15-day solids retention time (SRT) for a period of 6 months prior to the observations reported here.

### 2.2. Experimental design

All samples were prepared in a 1-m<sup>3</sup> Plexiglas chamber under a 99.9% N<sub>2</sub> headspace in which the absence of oxygen was continuously verified using disposable colorimetric indicators (Sigma, St. Louis, MO). Anaerobic digester filtrate was prepared by centrifuging (3500 rpm) digester sludge from the bench-scale digesters for 20 min and filter-sterilizing (0.2  $\mu$ M) the centrate two times. Samples included (i) filtrate amended with anaerobic digester biomass (1:10 (v/v)); (ii) cell-free filtrate only; (iii) cell-free filtrate amended with proteinase K; (iv) oxygenated cell-free filtrate; (v) oxygenated cell-free filtrate amended with proteinase K; and (vi) ultrapure water. A 5 mM CTC (final concentration) was added to each sample followed by a 15-h incubation on a shaker table at room temperature in the dark. For comparison of FISH and CTC assays as indicators of activity, digesting sludge (75 ml) was pipetted into serum vials that served as easily replicated digester microcosms in accordance with previously reported methods (Gruden et al., 2001). Statistical comparisons between samples were determined using *t*-tests ( $\alpha = 0.05$ ) in Microsoft Excel (Seattle, WA).

### 2.3. Dehydrogenase activity stain

The metabolic activity of the anaerobic digester sludge was determined using the tetrazolium redox dye 5-cyano-2,3-ditolylyl tetrazolium chloride (CTC; Polyscience, Warrington, PA). CTC assays were performed using a procedure modified from Rodriguez et al. (1992). Filter-sterilized (0.2  $\mu$ m) CTC stock solution (20 mM) was prepared in ultrapure water and stored at 4 °C for a maximum of 5 days. The appropriate final concentration of CTC was determined in an optimization experiment where CTC concentrations of between 2 and 10 mM were tested similar to methods previously reported (Bhupathiraju et al., 1999; Smith et al., 1994). Contrary to other reports in the literature (Ullrich et al., 1996), toxicity, identified as a statistically significant change in CTC reduction, was not observed between 5 and 10 mM CTC in active digester sludge. After incubation, dilutions of digester sludge were filtered through a 0.2- $\mu$ m black polycarbonate filter (Poretics, Cat. No. K02BP02500) backed with a 5- $\mu$ m silver membrane (Osmonics, Cat. No.46680). Filters were dried and mounted on a slide with immersion oil and coverslip. In some instances, a CTF extraction method, modified from Bhupathiraju et al. (1999) and Fonseca et al. (2001), was used. Following CTC incubation, the samples were centrifuged for 10 min at 1700 rpm and amended with HPLC grade ethyl acetate (100%) and mixed thoroughly. After a 30-min incubation period, absorbance of the sample centrate was measured at 450 nm.

### 2.4. CTC reduction spectra

A Hanging Mercury Drop electrode (HMDE) polarograph (CV-50, BAS systems, West Lafayette, IN), set to 30-s runs at 25 mV/s between 0 and  $-1.3$  V, was used to determine the reduction potential of CTC under ionic strength and pH conditions similar to that of anaerobic digester biomass. A 5 mM solution of CTC was used in an electrolytic solution of 0.85% NaCl (pH = 7). Oxidation–reduction spectra for CTC solutions were obtained in triplicate.

### 2.5. Image analysis

The microscope-image analysis system consisted of an Eclipse E400 Microscope (Nikon, Tokyo,

Japan), 24-bit cooled color digital camera (Spot Camera, Diagnostic Instruments, Sterling Heights, MI), C-Imaging Simple PCI software (Compix, Imaging Systems, Cranberry Township, PA), and a Pentium II-based personal computer. The microscope was equipped with an epifluorescence attachment (Y-FL, Nikon), a super high-pressure mercury lamp power supply (HB-10103AF, Nikon), and a 100-W mercury lamp (USH-102DH; S&M Microscopes, Colorado Springs, CO) for epifluorescence illumination. The following excitation and emission filter sets were chosen in accordance with fluorescent dye manufacturers' recommendations: CTC—exciter HQ545/30; emission HQ610/75; beamsplitter Q565LP; DAPI—exciter D360/40 and beamsplitter 406DCLP. The color camera detected the images and integrated software provided gain controls, manual or automatic black level, and color balance adjustments. For the analyses described in this work, only a single image color (red) was used.

The Simple PCI software was used for image analysis of CTC stained biomass. The total area (AREA) of the CTF found in each field was defined by a minimum intensity threshold of red color, and then selected by the image analysis software. Simple PCI measured the selected red areas in picture elements (pixels). The average intensity (MEAN RED) of the identified CTF area was also quantified. The product of MEAN RED and AREA was defined as MASS. At least 10 fields of each slide were analyzed. Standard deviations of less than 30% were deemed acceptable for AREA and MEAN RED quantities. CTF quantities were reported in reference to total biomass based on total counts (see below). The method detection limit for the CTC activity assay ( $\text{MASS} = 5.50 \times 10^5$ ) was defined as the smallest value that can be reliably detected above the random background noise found in analytical blanks and was determined statistically (Berthoux and Brown, 1994).

## 2.6. FISH

Two oligonucleotide probes, circumscribing the domains *Archaea* (ARC915) and *Bacteria* (EUB338), were used for direct microscopic FISH analysis on digesting sludge samples. These oligonucleotides (Genosys Biotechnologies, Woodlands, TX) have been well characterized and used successfully in many

environments including anaerobic digesters (Gruden et al., 2001; Merkel et al., 1999; Raskin et al., 1994a,b). The probe ARC915 targets all *Archaea* and was chosen for this work since the environmental conditions did not favor the growth of nonmethanogenic archaea such as extreme halophiles and thermoacidophiles (Raskin et al., 1994b). While the true ecology of acidogenic microorganisms has not been comprehensively described, the domain-level EUB338 probe was used to measure the activity of other nonmethanogenic microorganisms present in the digesters, including acidogenic populations (Gruden et al., 2001; Merkel et al., 1999). A fluorescent DNA intercalating agent, 4',6-diamino-2-phenylindole (DAPI, Sigma), was used to concurrently determine the total number of cells present regardless of their ecology or activity.

The fixation, filtration, and enumeration methods, as well as the detection limit used for the FISH analysis in this research, were adapted from widely accepted protocols (Raskin et al., 1994a,b) and were previously reported (Gruden et al., 2001).

## 3. Results

The reduction potential(s) of CTC was determined under pH and ionic strength conditions similar to anaerobic digester environments using differential pulse polarography. CTC reduction commenced at  $-50$  mV and exhibited two peaks corresponding to independent electron transfers completed at  $-175$  and  $-400$  mV (Fig. 1). A third maximum occurred near  $-1300$  mV, but was omitted from the figure since this redox condition is not common to natural or engineered environments. Fig. 2 illustrates the results of solvent-extracted CTF samples measured using a spectrophotometer. The absorbance (450 nm) of oxygenated cell-free filtrate samples was similar to the ultrapure water samples. A statistically significant increase in CTF was measured in all other samples as compared to the ultrapure water control. The cell-free filtrate samples amended with proteinase K resulted in an absorbance significantly lower than in otherwise identical cell-free filtrate. There was no statistically significant difference between absorbance of the biomass-amended filtrate and cell-free filtrate.

CTC reduction was observed by epifluorescent microscopy ( $1000\times$ ) in the cell-free filtrate and

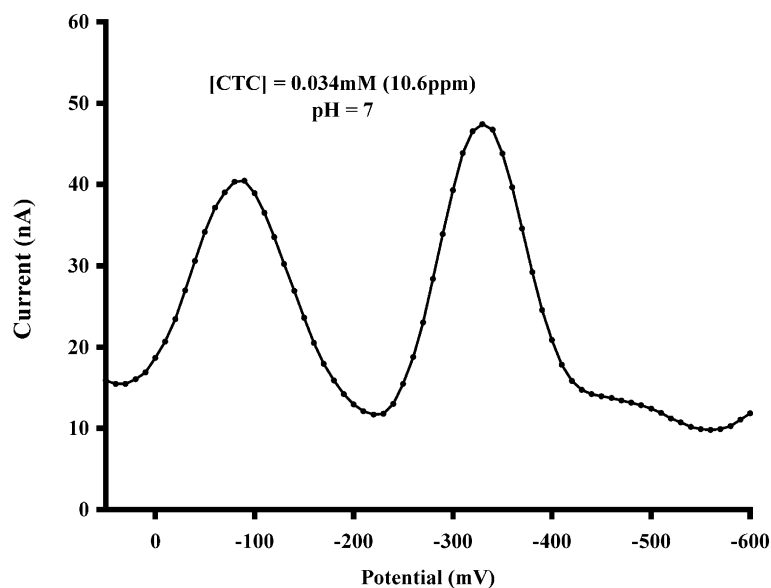


Fig. 1. CTC reduction spectra determined by differential pulse polarography under ionic strength and pH conditions similar to that of anaerobic digester filtrate tested in this study. Independent electron transfers were maximum near  $-80$  and  $-340$  mV. A third maxima occurred near  $-1300$  mV (data not shown).

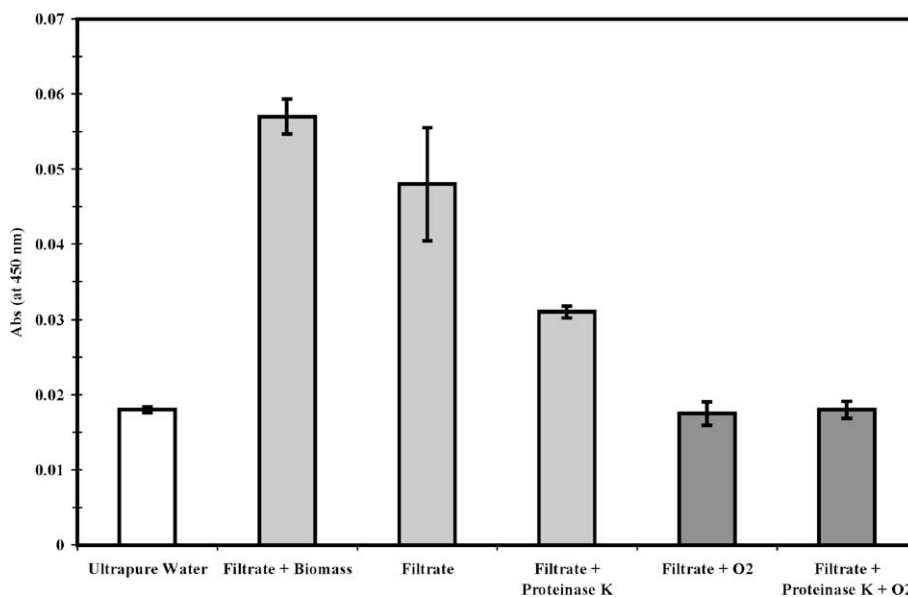


Fig. 2. Spectrophotometric absorbance ( $\lambda=450$  nm) as a function of sample type. Each sample included twice-filtered ( $0.2 \mu\text{m}$ ) anaerobic digester sludge (filtrate). Error bars represent  $\pm$  one standard error,  $n=3$  replicates.

biomass-amended filtrate samples (Figs. 3 and 4). The precipitate generated from the filtrate (only) sample appeared crystalline in nature with sharply defined edges; these precipitates exhibited a uniform intensity. When amended with biomass, the filtrate typically contained both rod-shaped bacteria with intracellular, localized CTC precipitate, as well as the extracellular CTC crystals previously described.

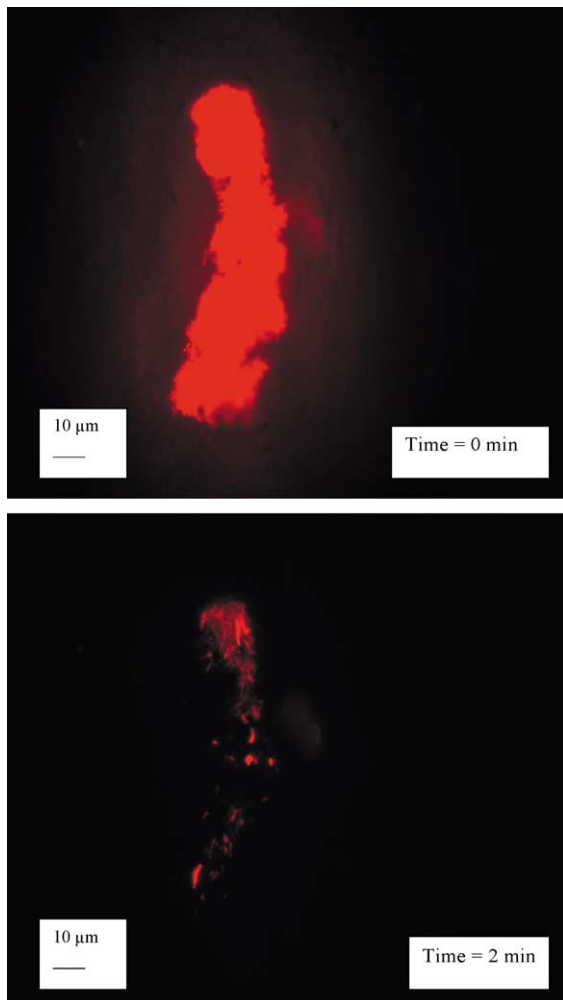


Fig. 3. Example of CTC reduction products formed in the absence of bacterial cells in filter sterilized (0.2 µm) anaerobic digester centrate. Top panel and bottom panel are microscope photographs (1000×) of the same CTC–Formazan (CTF) precipitates taken 2 min apart under constant UV irradiation. The bleaching shown is typical of the rapid photodegradation of these non-cell-associated CTC precipitates. Note relatively uniform fluorescence of CTC.

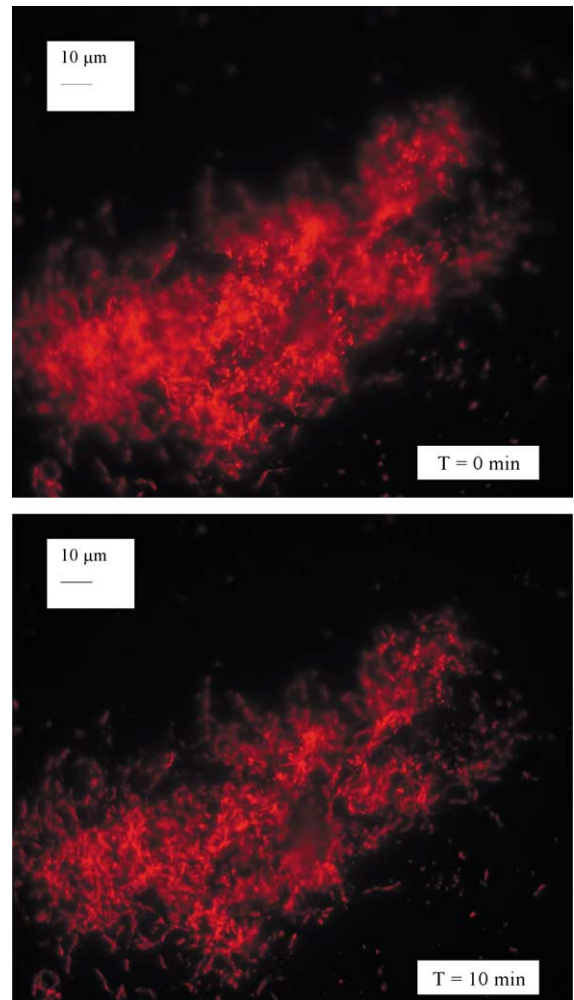


Fig. 4. Example of CTC reduction products formed in the presence of bacterial cells in filter sterilized (0.2 µm) anaerobic digester centrate. Top panel and bottom panel are microscope photographs (1000×) of the same CTC–Formazan (CTF) precipitates taken 10 min apart under constant UV irradiation. The bleaching shown is typical of the relatively slow photodegradation of these cell-associated CTC precipitates.

Fig. 5 demonstrates the MASS decrease of cell-free filtrate with respect to CTC formed in the presence of active cells (digester biomass) in response to UV exposure. The CTC MASS in the cell-free filtrate experienced exponential decline ( $y = 0.60e^{-1.70x}$ ,  $R^2 = 0.92$ ) to background levels within 2 min. This decline could be modeled as 1st order decay with a rate constant of approximately 1.7/min. A statistically significant decrease in CTC MASS of cell-free filtrate

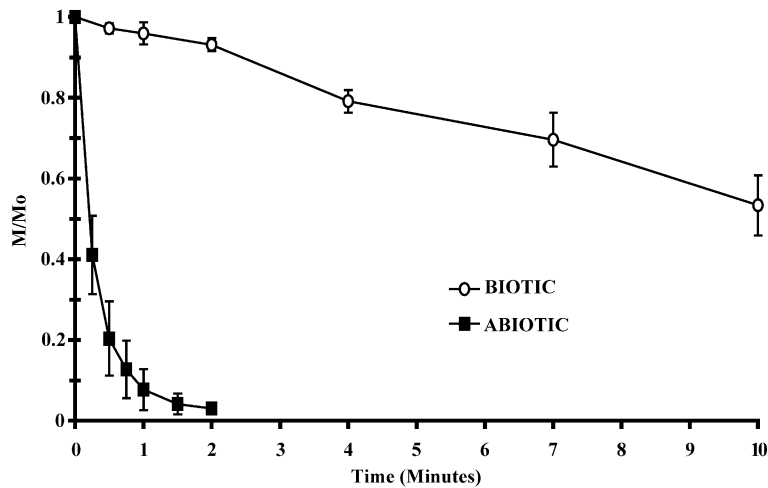


Fig. 5. Relative MASS ( $M/M_0$ ) as a function of UV exposure. MASS is equivalent to Red Area  $\times$  Mean Red Pixel Intensity. Timed experiment for dehydrogenase activity assay (CTC staining technique). Red Area and Mean Red Pixel Intensity were measured using an Eclipse E400 Microscope (Nikon), 24-bit cooled color digital camera (Spot Camera, Diagnostic Instruments), C-Imaging Simple PCI software (Compix, Imaging Systems), and a Pentium II-based personal computer. Error bars represent  $\pm$  one standard error,  $n=3$  replicates.

occurred in less than 30 s. The CTF MASS of the filtrate containing active cells decreased linearly ( $y = -0.05x + 1.00$ ,  $R^2 = 0.99$ ) and did not show a statistically significant decrease until after 4 min of UV exposure. After 10 min of UV exposure, the CTF

MASS in the presence of digester biomass decreased approximately 40%.

The dehydrogenase activity assays reported above and FISH were carried out simultaneously in microcosms containing anaerobic digesting biomass, pro-

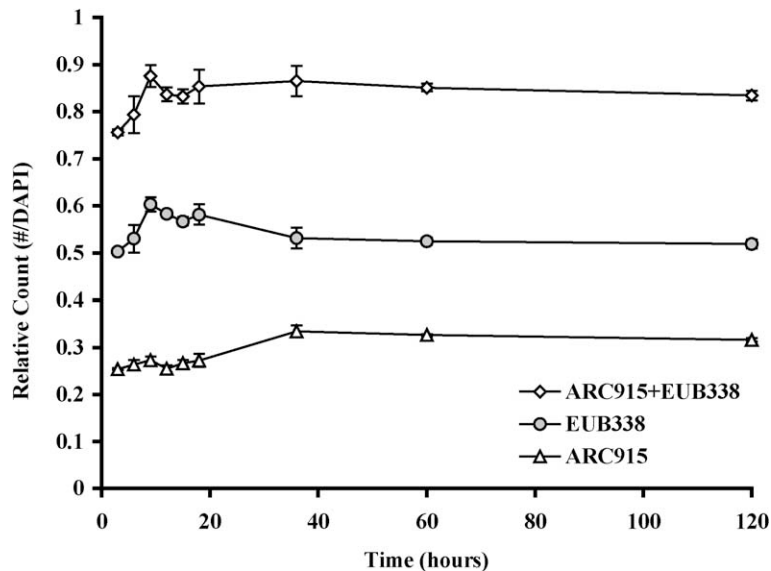


Fig. 6. Relative count as a function of time (hours). Direct counts of active digester bacteria hybridized with fluorescent genetic probes (FISH). FISH counts are normalized to total cell counts determined by DNA intercalating agents (DAPI). Temperature was  $35 \pm 2$  °C. Error bars represent  $\pm$  one standard error,  $n=3$  replicates.

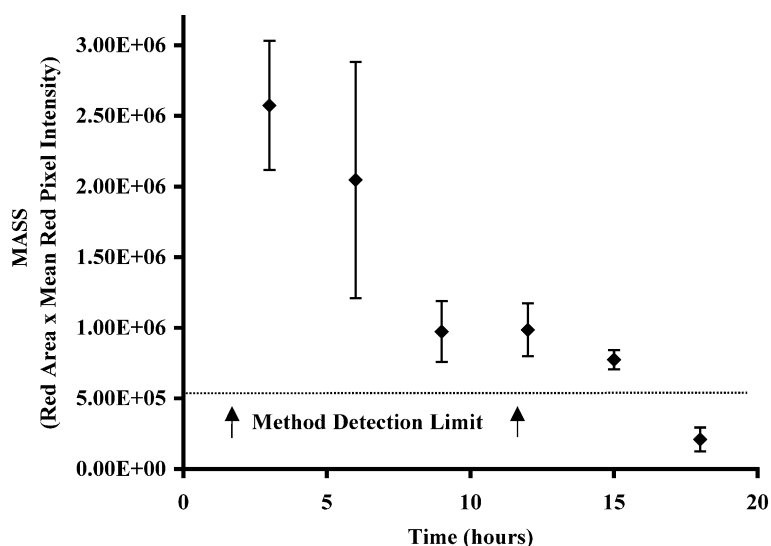


Fig. 7. MASS (Red Area  $\times$  Mean Red Pixel Intensity) as a function of time (hours). Timed experiment for dehydrogenase activity assay (CTC staining technique). Red Area and Mean Red Pixel Intensity were measured using an Eclipse E400 Microscope (Nikon), 24-bit cooled color digital camera (Spot Camera, Diagnostic Instruments), C-Imaging Simple PCI software (Compix, Imaging Systems), and a Pentium II-based personal computer. Temperature was  $35 \pm 2$  °C. Error bars represent  $\pm$  one standard error,  $n=3$  replicates.

pylene glycol, and settled sewage centrate for direct comparison. The number of active bacteria measured as determined by FISH remained well above the method detection limit for 5 days (Fig. 6). However, the signal recorded from dehydrogenase activity assay (CTC) decreased to below the method detection limit after 14 h (Fig. 7) of incubation.

#### 4. Discussion

The midpoint reduction potential (approximately  $-175$  mV) and CTF production at redox potentials as low as  $-50$  mV observed in this work correspond with previously published data under similar conditions (Smith and McFeters, 1997). Previous research indicates that CTC undergoes tetrazolanyl-radical cation intermediate stage during its reduction, which can result in a diffuse, poorly localized intercellular formazan at redox potentials higher than  $-200$  mV (Smith and McFeters, 1997). A secondary peak near  $-400$  mV, which has not been previously reported, suggests that CTC may be useful as a redox potential indicator under very low redox conditions such as those commonly encountered in anaerobic digesters.

CTC reduction occurred in cell-free anaerobic digester filtrate. This reduction may be due to extracellular enzymes (Lopez-Amoros et al., 1995), fragments of cell membranes (Smith and McFeters, 1997), or soluble reducing agents (Rodriguez et al., 1992; Bhupathiraju et al., 1999) in the anaerobic matrix that have been previously reported. Oxygenated cell-free filtrate samples did not produce measurable CTC reduction, presumably due to the preferential reduction of oxygen. The statistically significant decrease in CTC reduction with the addition of proteinase K indicates a role for enzymes in “abiotic” reduction of CTC. However, the CTC reduction (above background levels) observed in samples containing proteinase K suggests the presence of other reducing agents as previously observed in other anaerobic environments (Bhupathiraju et al., 1999; Smith and McFeters, 1997).

CTC reduction products, clearly identified as CTF in cell-free filtrate, were distinguished by epifluorescent microscopy ( $1000\times$ ) with a distinct crystalline morphology and uniform fluorescence (Fig. 3). Non-cellular-associated CTF dissipated quickly under UV-exposure intensities commonly used for epifluorescent microscopy ( $<2$  min). Care must be taken when

employing CTC for determination of metabolic activity in anaerobic digesters. Abiotic reduction may impede accurate enzyme activity measurements, particularly when solvent extraction or software-aided counting techniques are involved. Specific procedures including UV exposure (minimum 30 s) and discernment or morphological distinctions between cell-free and biotic formation can mitigate inaccurate measurements.

In the context of activity assays, a primary objective of this research was to compare the response of CTC and quantitative fluorescent in situ hybridization (FISH) with phylogenetic probes for metabolic activity assessment. The period of observation required to obtain useful data from each of these assays was markedly different. Microcosms were amended with a single input of primary sludge centrate (3000 mg/l COD), which was readily biodegradable. Quantitative FISH measurements remained well above the detection limit for the populations targeted indicating sustained metabolism (rRNA content) over a 5-day period (Fig. 6), while dehydrogenase assays (CTC) decreased to background levels within 14 h (Fig. 7), which would suggest substrate limitation. Starving cells may carry out biosynthetic processes (protein production) while not actively growing, or maintain rRNA content as an endogenous process. Results from this study correspond to those of Sherr et al. (1999), who reported CTC-negative cells in a bacterioplankton assemblage that were identified as active via indices of cell-specific rRNA content. Under such circumstances, CTC reduction may be ceased or CTF production may exist below the method detection limit (del Giorgio et al., 1997). This hypothesis was supported by Schaule et al. (1993), who observed a dramatic decrease in CTF production after only 5 min of starvation when working with *P. putida* in biofilms. Further, Walsh et al. (1995) determined that CTC successfully determined metabolic activity of a sulfate reducing culture during exponential growth; however, microscopy underestimated the numbers of starved but metabolically active cells (after 25 h of incubation).

In this study, extracellular reduction of CTC was confirmed in anaerobic digester sludge filtrate. This reduction may be attributed to extracellular enzymes and chemical reductants present in the filtrate. These results suggest that both depressed redox condition (ca.  $< -50$  mV), as well as the presence of extracellu-

lar enzymes, can contribute to the extracellular reduction of CTC to CTF, and that an abiotic auto-reduction of CTC can occur under reducing conditions common to anaerobic digesters (ca.  $< -200$  mV). Further, the structure of CTF can vary such that its photostability is significantly different when formed inside or outside active microbiological cells.

Care must be taken when measuring CTC reduction in anaerobic samples, particularly when using solvent extraction or software-aided quantification. Epifluorescent microscopic observation ( $1000\times$ ) of the fluorescent precipitate may allow distinction between intracellular and extracellular CTF. Also, UV exposure may result in rapid degradation ( $< 2$  min) of extracellular CTF. Activity assessment using FISH and CTC reduction assays may be comparable until substrate becomes limited.

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