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Determination of microbial respiratory and redox activity in activated sludge

T Griebe[1], G Schaule[2] and S Wuertz[3]

[1]*Gerhard-Mercator-Universität-GH Duisburg, Aquatische Mikrobiologie;* [2]*Institut für Wasserchemie und Wassertechnologie (IWW), Institut an der Gerhard-Mercator-Universität-GH Duisburg, Moritzstr 26, D-45476 Mülheim/Ruhr;* [3]*Lehrstuhl für Wassergüte- und Abfallwirtschaft der TU München, Am Coulombwall, D-85748 Gatching, Germany*

The tetrazolium salt 5-cyano-2,3-ditolyltetrazolium chloride (CTC) was used for the determination of metabolically active bacteria in active sludge. The method was adapted and optimized to the conditions of activated sludge. The colorless and nonfluorescent tetrazolium salt is readily reduced to a water-insoluble fluorescent formazan product via the microbial electron transport system and indicates mainly dehydrogenase activity. After more than 2 h incubation, no further formation of new formazan crystals was observed, although the existing crystals in active cells continued to grow at the optimal CTC-concentration of 4 mM. The dehydrogenase activity determined by direct epifluorescence microscopic enumeration did not correlate with cumulative measured activity as determined by formazan extraction. The addition of nutrients did not lead to an increase of CTC-active cells. Sample storage conditions such as low temperature or aeration resulted in a significant decrease in dehydrogenase activity within 30 min. The rapid and sensitive method is well suited for the detection and enumeration of metabolically active microorganisms in activated sludge. Extracellular redox activity was measured with the tetrazolium salt 3′-{1-[phenylamino-) carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT), which remains soluble in its reduced state, after extraction of extracellular polymeric substances (EPS) with a cation exchange resin.

Keywords: CTC; activated sludge; bacterial activity; formazan; tetrazolium salt; waste water treatment

Introduction

Determination of microbial activity is of importance for process analysis in wastewater because the degradation of organic matter, system productivity, and biomass turnover all depend on metabolically active microorganisms. The activity of bacteria in activated sludge is usually evaluated by measuring general parameters such as rate of oxygen uptake, adenosine triphosphate (ATP) content, biomass, or substrate utilization. These parameters do not provide any information about the distribution of microbial activity or viability in sludge populations. As of yet it is not known which bacteria in activated sludge are metabolically active and what constitutes their impact on system performance. Plate count procedures are time consuming and considerably underestimate the number of viable bacterial cells in activated sludge. Attempts to measure active cell mass in activated sludge using tetrazolium salts have been made by several researchers. Since all anaerobic and aerobic heterotrophic bacteria possess electron transport systems, tetrazolium salts like triphenyltetrazolium chloride (TTC) and 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) can be used as artificial electron acceptors to detect dehydrogenase activity and thus metabolically active bacteria [2,5]. The detection of activity is based on the reduction of the water-soluble and colorless tetrazolium salts to colored crystals of the water-insoluble formazan products. The tetrazolium salt TTC is reduced to a red insoluble formazan which can be extracted from cells and measured colorimetrically; this has been used as a measure of cumulative activity in activated sludge [3,13,14]. Similar approaches were made using the tetrazolium salt INT [1,6,7]. The nonfluorescent formazan crystals of the tetrazolium salts TTC and INT are difficult to detect microscopically and thus, have limited general use. In contrast, the fluorescent formazan of the redox-sensitive dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is easily detected by epifluorescence microscopy [9,11,12]. Physiologically active bacteria are distinguishable from other particles in activated sludge because of their bright red fluorescence. Furthermore, the formazan deposits of CTC are extractable with ethanol for spectrophotometric quantification.

In this study, the effect of different incubation procedures and CTC concentrations on the formation of formazan in activated sludge was examined. Extracellular redox activity was measured with the tetrazolium salt 3′-{1-[(phenylamino-)carbonyl]3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) which produces a water-soluble formazan salt by following its reduction in extracellular extracts containing polymeric substances (EPS). Furthermore, the effect of sample storage on dehydrogenase activity was evaluated.

Materials and methods

A flow diagram of the dehydrogenase activity assay is presented in Figure 1. Activated sludge samples were incubated with different CTC concentrations (1–10 mM) at room temperature in the dark.

The tetrazolium salts 5-cyano-2,3-ditolyltetrazolium chloride (CTC) and 3′-{1-[(phenylamino-)carbonyl]-3,4-

Correspondence: Dr T Griebe, Gerhard-Mercator-Universität-GH Duisburg, Aquatische Microbiologie, IWW, Moritzstrasse 26, 45476 Mülheim, Germany
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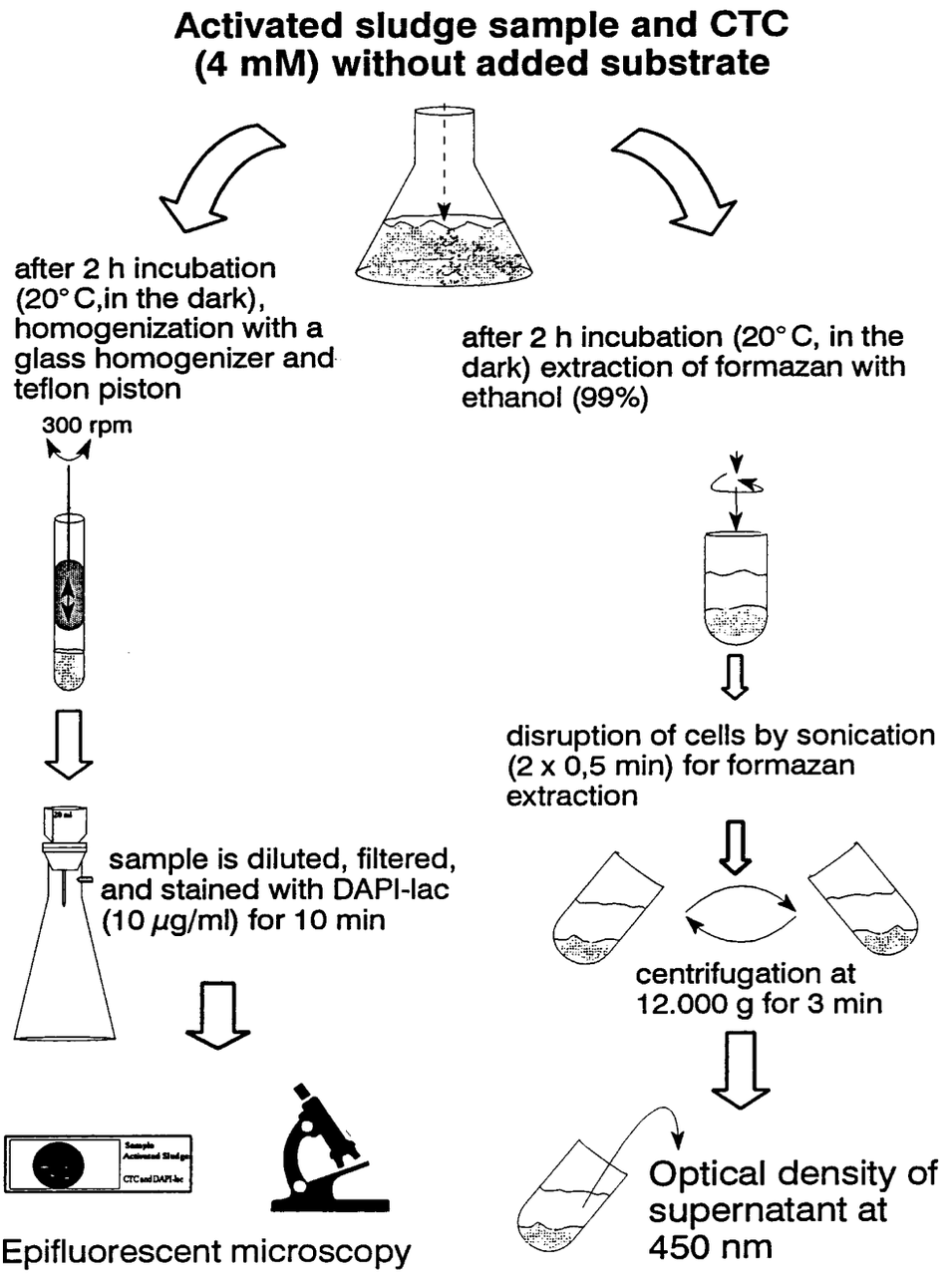

Figure 1 Flow diagram of the dehydrogenase activity assay in activated sludge.

tetrazolium}-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) and the DNA-binding fluorochrome 4,6,-diamidino-2-phenylindoldihydrochloride (DAPI) were purchased from Polyscience Inc (Eppelheim, Germany). The tetrazolium salt triphenyltetrazolium chloride (TTC) and the corresponding 1,3,5-triphenyl-tetrazoliumformazan were purchased from Sigma (Deisenhofen, Germany). The formazan salt of XTT was synthesized by ascorbic acid reduction of pure XTT [8]. Controls were prepared by incubating samples previously inactivated by formaldehyde (final conc. 2% vol/vol). After incubation (2–24 h), samples were counterstained with DAPI (10 μg ml^{-1}) for determination of total cells. Activated sludge samples were homogenized and diluted prior to filtration onto a black 0.2-μm pore-size polycarbonate membrane filter (Millipore, Ireland). Appropriate disaggregation of the actived sludge matrix without disruption of bacterial cells was achieved in a 10-ml glass homogenizer with a teflon piston at 300 rpm for 4 min. Microscopic examination and counting of triplicate samples were done according to Schaule *et al* [11]. The overall dehydrogenase activity of activated sludge was determined spectrophotometrically after extraction of the formazan with ethanol. Sludge samples incubated up to 24 h with 4 mM CTC at room temperature were centrifuged at 12 000 $\times$ *g* for 3 min. The pellets were resuspended in 99% ethanol and disrupted by sonication. After centrifugation the supernatant was collected and the pellets extracted for a second time with ethanol. The absorbance of the col-

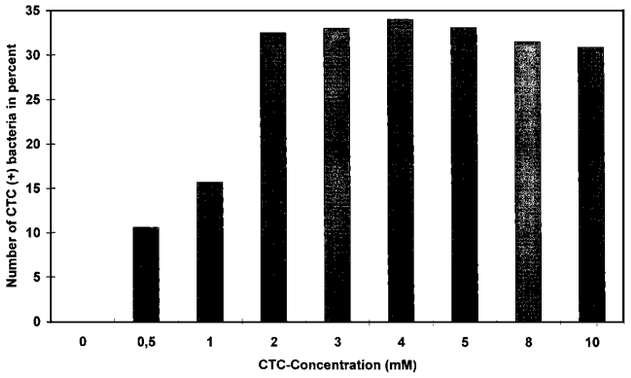

Figure 2 Influence of CTC concentration on dehydrogenase activity in activated sludge. Activity is expressed as percent CTC-positive bacteria of total bacteria determined by epifluorescence microscopy.

lected supernatants was determined at a wavelength of 450 nm. Extracellular redox activity was measured by extracting extracellular polymeric substances (EPS) as previously described [4] and following the reduction of CTC, TTC and XTT to their respective formazan salts. The absorbance was measured after a 24-h incubation period. In the case of CTC and TTC the EPS were centrifuged at $20\,000 \times g$ for 15 min at 4°C to precipitate formazan crystals and the precipitate was extracted with ethanol (99%). The absorbance of the resulting solution was measured at a wavelength of 450 (CTC-formazan) or 480 nm (TTC-formazan). In the case of XTT no extraction of crystals was necessary since the formazan is water-soluble. The absorbance of XTT-formazan was determined at 470 nm [10] and compared to total XTT activity in sludge samples.

Results

The formazan deposition in activated sludge bacteria was dependent on CTC up to a concentration of 2 mM during a 2-h incubation time (Figure 2). The optimal CTC concentration for the detection of formazan crystals in activated sludge bacteria using epifluorescence microscopy was in the range of 2–4 mM.

Supplementation of activated sludge samples with R2A medium [9] or acetate/nitrate did not result in a substantial increase in the number of CTC-positive bacteria in activated sludge during a 2-h incubation (results not shown).

A high CTC concentration (10 mM) resulted in slightly but not significantly reduced CTC-formazan deposition inside the bacteria as estimated by epifluorescence microscopy (Table 1). The activity is expressed as percent CTC-positive bacteria of total bacteria determined by epifluorescence microscopy or as optical density at 450 nm of formazan extracts.

In contrast, the measurement of cumulative dehydrogenase activity by extractable formazan showed a significantly reduced formation of fluorescent formazan deposits at a CTC concentration of 10 mM as determined by one-way analysis of variance with a level of significance set at $P < 0.05$ for all comparisons (Table 1). The decreased reduction of CTC at high concentration (10 mM) indicates physiological inhibition located in the electron transport system. Furthermore, it is shown that the amount of extractable formazan is not correlated with the number of physiologically active bacteria. To determine the optimal incubation time for the dehydrogenase activity assay, activated sludge was incubated over a period of 24 h with 4 mM CTC. After 2 h incubation time, no further increase in the number of CTC-positive bacteria was found, but larger crystals were observed with increasing incubation time (Figure 3). It is difficult to compare microscopic counts with photometric results since the size of formazan crystals inside the bacteria increased during the incubation time of 24 h as viewed by microscopy and measured spectrophotometrically (Figure 4).

In order to evaluate the effect of sample storage on dehydrogenase activity, activated sludge samples were aerated at 20°C, stirred at 20°C, or cooled at 4°C. After short periods aliquots were taken for dehydrogenase activity measurements. Regardless of the storage method, the activity decreased about 30% within the first 60 min (Figure 5). These results clearly show that any kind of sample storage has to be avoided when determining microbial activity in activated sludge. Usually the transport of sludge to the laboratory and subsequent analysis require 1–2 h. Considering the dramatic change in activity due to storage it is essential to incubate the sample with CTC at the sampling location.

In order to substantiate the view that dehydrogenase activity occurred extracellularly as well as intracellularly, EPS were extracted using an ion exchange resin. The resulting formazan crystals accounted for almost half the total activity as measured by reduction of XTT (Table 2).

Table 1 Influence of CTC concentration on dehydrogenase activity in activated sludge[a]

Method for activity determination	CTC-concentration	X	s.d.	*n*
Cellular (microscopy)	4 mM	22.50%	3.00%	4
Cellular (microscopy)	10 mM	17.75%	2.75%	4
Cumulative (photometry)	4 mM	0.506[b]	0.027	8
Cumulative (photometry)	10 mM	0.364[b]	0.056	8

[a]X = Mean value of CTC-positive bacteria or extracted formazan; s.d. = standard deviation; *n* = number of samples.
[b]Means are significantly different from one another at $P < 0.05$.

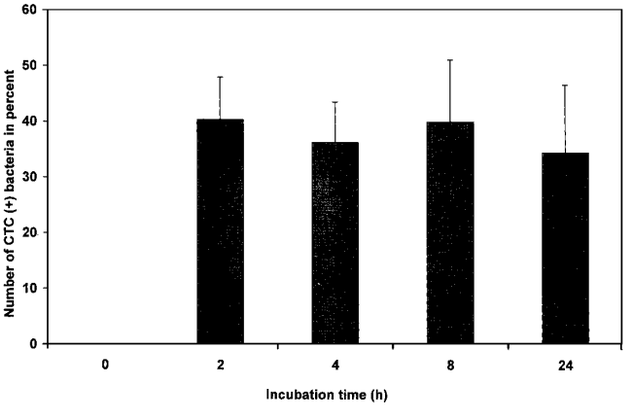

Figure 3 Influence of incubation time on dehydrogenase activity in activated sludge. Activity is expressed as percent CTC-positive bacteria of total bacteria determined by epifluorescence microscopy.

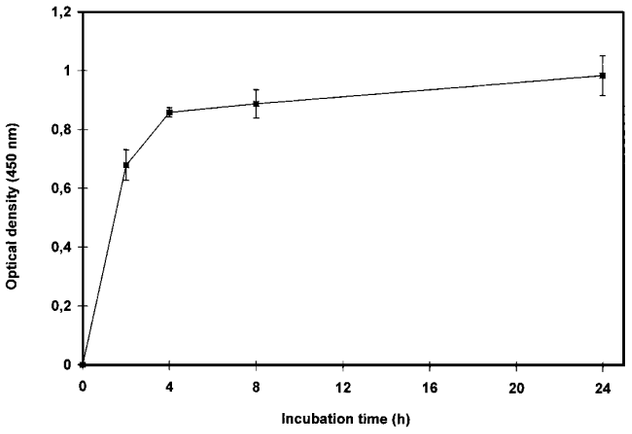

Figure 4 Influence of incubation time on dehydrogenase activity in activated sludge. Cumulative dehydrogenase activity after formazan extraction with ethanol was measured spectrophotometrically at a wavelength of 450 nm.

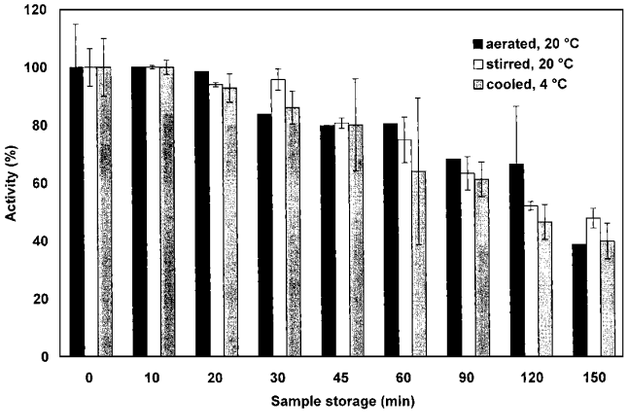

Figure 5 Impact of storage conditions (aeration 20°C, stirring at 20°C, or cooling at 4°C) on dehydrogenase activity in activated sludge. Activity is expressed as percent decrease compared to incubation on site.

Table 2 Reduction of XTT and TTC to formazan by activated sludge

Tetrazolium salt	Formazan production[a] (μg mg^{-1} dry weight)		*n*
	Total	Extracellular	
XTT	136.47 (±4.00)	66.90 (±6.7)	2
TTC	24.39	0.17 (±0.03)	2

[a]±SEM.

In contrast, less TTC was reduced, and the extracellular activity as determined by TTC-formazan production was less than 1% of total activity. Further details about extracellular redox activity will be provided in a separate publication [15].

Discussion

It is noteworthy that the two methods for determination of physiological activity in sludge lead to inconsistent results. The cumulative measured activity using ethanol extraction of formazan deposits is a rapid method but has limited application because it is obviously not correlated with the number of physiologically active microorganisms found in activated sludge. Miroscopic observation showed that CTC-formazan deposits were found in the activated floc matrix outside bacterial cells. Since the sterile control did not show any formazan deposits it is concluded that bacterial cell components and redox enzymes attributed to the floc matrix facilitate the reduction of the tetrazolium salt extracellularly. This view is supported by the observed reduction of XTT in extracellular extracts. An extracellular reduction of CTC was not shown, and the reduction of TTC was minor, but this can be attributed to methodological difficulties, ie the number of crystals was too small to allow efficient precipitation prior to ethanol extraction. INT and TTC are more readily reduced than CTC, and TTC-formazan was produced in extracellular extracts. Therefore, measurements of dehydrogenase activity determined with INT and TTC are misleading because the number of active bacteria is not correlated with the amount of intracellular and extracellular formazan deposits in sludge samples. However, most researchers using TTC or INT for activity measurements in activated sludge do not mention extracellular formazan formation.

The *in situ* enumeration method with the tetrazolium salt CTC provides rapid (within 2 h) and precise (detection of a single active bacterium) information regarding activated sludge composition and its current physiological state. Furthermore, the CTC method constitutes a convenient and rapid approach for quantification and monitoring of inhibitory effects in activated sludge treatment processes.

The results showed that CTC is well suited for measurements of microbial activity in activated sludge. The period between sampling and measurements has a strong influence on the obtained results even in the range of minutes. Against this background, literature data on microbial activity in activated sludge have to be critically re-evaluated with respect to the time lapsed before samples were measured.

Acknowledgements

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