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Development of a Rapid Ferricyanide-Mediated Assay for Biochemical Oxygen Demand Using a Mixed Microbial Consortium

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Ferricyanide-mediated (FM) microbial reactions were used for the rapid determination of the biochemical oxygen demand (BOD) of a range of synthetic and real wastewater samples. Four single-species microbial seeds and a synthetically prepared microbial consortium were compared. In all cases, the microbial consortium exhibited a greater extent and rate of biodegradation compared to the individual microbial seeds. Markedly improved correlation to the standard BOD_5 method was also noted for the microbial consortium (compared to the single-species seeds). A linear dynamic range up to 200 mg BOD_5 L^{-1} was observed, which is considerably greater than the linear range of the standard BOD_5 assay and most other rapid BOD assays reported. In addition, biodegradation efficiencies comparable to the 5-day BOD_5 assay (and much greater than other rapid BOD assays) were observed in 3 h. A highly significant correlation ($R = 0.935$, $p = 0.000$, $n = 30$) between the FM-BOD method and the standard BOD_5 method was found for a wide diversity of real wastewater samples. The results indicate that the FM-BOD assay is a promising, rapid, alternative to the standard 5-day BOD_5 assay.

The development of rapid methods for the determination of biodegradable organic pollutants in waters and wastewaters has received considerable attention in recent years.[1−26] The current standard method for determining biodegradable organic compounds in wastewaters is the 5-day biochemical oxygen demand (BOD_5) assay. This test quantifies the amount of dissolved oxygen required for the microbial oxidation of organic material (carbonaceous demand) in 5 days under specified conditions.[27,28] Apart from taking 5 days to complete, it also involves tedious, time-consuming procedures and requires dedicated laboratory facilities and a high level of skill and experience to obtain accurate, reproducible results.[29] In addition, the limited linear working range of the BOD_5 method (1−8 mg/L O_2), which results from poor O_2 solubility, means that excessive dilution of the sample is generally required.

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(1) Karube, I.; Matsunaga, T.; Suzuki, S. *J. Solid-Phase Biochem.* **1977**, *2* (2), 97−104.

(2) Karube, I.; Mitsuda, S.; Matsunaga, T.; Suzuki, S. *J. Ferment. Technol.* **1977**, *55* (3), 243−248.

(3) Hikuma, M.; Suzuki, H.; Yatsuda, T.; Karube, I.; Suzuki, S. *Eur. J. Appl. Microbial. Biotechnol.* **1979**, *8*, 289−297.

(4) Harita, K.; Otani, Y.; Hikuma, M.; Yasuda, T. *Instrum. Control Water Wastewater Treat. Transp. Syst., Proc. IAWPRC Workshop, 4th* **1985,** 529−532.

(5) Riedel, K.; Renneberg, R.; Kuehn, M.; Scheller, F. *Appl. Microbiol. Biotechnol.* **1988**, *28* (3), 316−318.

(6) Riedel, K.; Lange, K. P.; Stein, H. J.; Kuhn, M.; Ott, P.; Scheller, F. *Water Res.* **1990**, *24*, 883−887.

(7) Ohki, A.; Shinohara, K.; Ito, O.; Naka, K.; Maeda, S. *Int. J. Environ. Anal. Chem.* **1994**, *56* (4), 261−269.

(8) Yang, Z.; Suzuki, H.; Sasaki, S.; Karube, I. *Appl. Microbiol. Biotechnol.* **1996**, *46* (1), 10−14.

(9) Yang, Z.; Sasaki, S.; Karube, I.; Suzuki, H. *Anal. Chim. Acta* **1997**, *357* (1−2), 41−49.

(10) Riedel, K.; Lehmann, M.; Tag, K.; Renneberg, R.; Kunze, G. *Anal. Lett.* **1998**, *31* (1), 1−12.

(11) Qian, Z.; Tan, T. C. *Water Res.* **1999**, *33* (13), 2923−2928.

(12) Tan, T. C.; Wu, C. *Sens. Actuators, B* **1999**, *54* (3), 252−260.

(13) Liu, J.; Bjornsson, L.; Mattiasson, B. *Biosens. Bioelectron.* **2000**, *14* (12), 883−893.

(14) Pasco, N.; Baronian, K.; Jeffries, C.; Hay, J. *Appl. Microbiol. Biotechnol.* **2000**, *53* (5), 613−618.

(15) Yoshida, N.; Yano, K.; Morita, T.; McNiven, S. J.; Nakamura, H.; Karube, I. *Analyst* **2000**, *125* (12), 2280−2284.

(16) Catterall, K.; Morris, K.; Gladman, C.; Zhao, H.; John, R. *Talanta* **2001**, *55*, 1187−1194.

(17) Morris, K.; Catterall, K.; Zhao, H.; Pasco, N.; John, R. *Anal. Chim. Acta* **2001**, *442* (1), 129−139.

(18) Yoshida, N.; McNiven, S. J.; Yoshida, A.; Morita, T.; Nakamura, H.; Karube, I. *Field Anal. Chem. Technol.* **2001**, *5* (5), 222−227.

(19) Yoshida, N.; Junko, H.; Morita, T.; McNiven, S. J.; Nakamura, H.; Karube, I. *J. Biotechnol.* **2001**, *88*, 269−275.

(20) Sakai, Y.; Abe, N.; Takeuchi, S.; Takahashi, F. *J. Ferment. Bioeng.* **1995**, *80* (3), 300−303.

(21) Chan, C.; Lehmann, M.; Tag, K.; Lung, M.; Kunze, G.; Riedel, K.; Gruendig, B.; Renneberg, R. *Biosens. Bioelectron.* **1999**, *14* (2), 131−138.

(22) Galindo, E.; Garcia, J. L.; Torres, L. G.; Quintero, R. *Biotechnol. Technol.* **1992**, *6* (5), 399−404.

(23) Li, F.; Tan, T. C.; Lee, Y. K. *Biosens. Bioelectron.* **1994**, *9* (3), 197−205.

(24) Riedel, K. Application of biosensors to environmental samples. In *Commercial biosensors: application to clinical, bioprocess, and environmental samples*; Ramsay, G., Ed.; John Wiley & Sons: New York, 1998; Vol. 148.

(25) Tan, T. C.; Li, F.; Neoh, K. G.; Lee, Y. K. *Sens. Actuators, B* **1992**, *8* (2), 167−172.

(26) Tan, T. C.; Qian, Z. *Sens. Actuators,* **1997**, *40* (1), 65−70.

(27) American Public Health Association *Standard Methods for the Examination of Water and Wastewater*, 19th ed.; American Public Health Association: Washington, DC, 1997.

(28) *Japanese Industrial Standard Committee Testing Methods for Industrial Wastewater*, JIS: Tokyo Japan. 1993; p 48.

(29) Nomura, Y.; Chee, G. J.; Karube, I. *Field Anal. Chem. Technol.* **1998**, *2* (6), 333−340.

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Despite these drawbacks, the BOD_5 method remains the most important measurable index of biodegradability. This is principally due to the fact that it is a bioassay, employing microorganisms as a biocatalyst in the oxidative degradation of organic material. In this respect, it attempts to mimic the biooxidative processes found in natural aquatic environments. Unfortunately, this simulation of natural conditions, with inherently low O_2 concentrations, contributes to the most significant limitation of the BOD_5 assay—its 5-day duration. Poor O_2 solubility (8.7 mg/L at 25 °C) means that low microbial populations and low organic concentrations (obtained by dilution) are required to ensure that O_2 does not limit the microbial reaction. It is this decrease in overall reaction kinetics, imposed by low O_2 solubility, that is the most significant factor contributing to the 5-day duration of the standard method.[30] If the solubility of O_2 were higher, increased microbial populations and higher organic pollutant concentrations could be employed, thereby increasing the rate of degradation and decreasing assay time.

To date, the development of biological-based rapid BOD assays has centered on the development of BOD biosensors. This approach, pioneered by Karube and co-workers,[1,2] involves immobilizing microbial cells on a membrane placed in close, intimate contact with an amperometric oxygen electrode. Microbial respiration (i.e., oxygen uptake) is directly measured by the oxygen probe and quantitatively related to the BOD of the sample. This rapid, but local, decrease in O_2 levels allows assay times ranging from 15 s [5] to 30 min[3,8,31]—a considerable improvement over the standard BOD_5 method. The problem with this approach, however, is that only a small portion of the biodegradable organics present in a sample is actually degraded during the short assay time—this, again, is linked to limited O_2 availability. Typically, less than 1% (in some cases much less) of the available carbon is degraded in a BOD biosensor assay, compared with >60% for the standard BOD_5 assay.[17] The major implication of this is that BOD biosensors will over-represent the most easily biodegradable portion of the total BOD since this is the most likely fraction to be degraded in the limited reaction time. In addition, the relative proportion of the readily biodegradable fraction will vary between different samples—thus necessitating constant recalibration of the BOD biosensor for different sample types.

Rapid and exhaustive biodegradation of organic material is difficult in systems limited by the supply of dissolved oxygen.[17] In recent studies,[14,16,17] we have demonstrated the potential of employing ferricyanide-mediated microbial reactions for rapid BOD analysis. Yoshida and co-workers[15,19] have also recently combined ferricyanide-mediated microbial reactions with a biosensor approach for rapid BOD analysis. In ferricyanide-mediated processes, oxygen (the natural electron acceptor in aerobic microbial catabolism) is replaced by an artificial electron acceptor—i.e., the ferricyanide ion. The microbial reaction still involves oxidation of organic material to CO_2 via glycolysis and the TCA cycle (eq 1a). The electrons derived from this process, however, are transferred down the electron transport system to reduce ferricyanide to ferrocyanide (eq 1b) instead of being transferred to O_2 to produce H_2O. The overall ferricyanide mediated process is depicted in eq 1c.

$$CH_2O + H_2O \xrightarrow{\text{glycolysis/TCA}} CO_2 + 4H^+ + 4e^- \quad (1a)$$

$$[Fe(CN)_6]^{3-} + e^- \xrightarrow{\text{ETS}} [Fe(CN)_6]^{4-} \quad (1b)$$

$$CH_2O + H_2O + 4[Fe(CN)_6]^{3-} \xrightarrow{\text{microorganisms}} CO_2 + 4H^+ + 4[Fe(CN)_6]^{4-} \quad (1c)$$

The most significant advantage of using ferricyanide in place of O_2 for rapid BOD assays is its high solubility. This allows for much higher microbial populations without the rapid (rate-limiting) depletion of the electron acceptor. Consequently, the time required to degrade significant amounts of organic material is greatly reduced. In previous studies, degradation efficiencies comparable to the 5-day BOD_5 assay for the glucose−glutamic acid (GGA) standard BOD solution were achieved within 1 h under appropriate conditions.[14,17,32,33] Moreover, the need for excessive dilution of samples, to ensure that the organic material (and not the electron acceptor) is rate limiting, was greatly reduced. Typical linear working ranges for the ferricyanide-mediated BOD assay are from 0 to 200−300 mg BOD_5 L^{-1} for the standard GGA solution[17,32,33]—this is appreciably greater than both the standard method and reported values for BOD biosensors (see ref 24 and references therein).

The importance of the microbial component in rapid BOD assays has been addressed in some detail in the recent literature.[16,24] Of high priority, is the need to select a microbial system capable of rapidly and fully degrading as wide a range of organic compounds as possible. For BOD biosensors, this has been achieved in two ways. One approach uses a mixed population of species derived from the activated sludge of wastewater treatment plants[13,20]—the idea being that the wide diversity of microbial species present should be able to degrade a wide range of compounds. The other approach has been to employ well-characterized, pure (single species) cultures of microorganisms with broad range substrate utilization.[1,3−7,9,21,23] Synthetically prepared combinations/consortia of these microorganisms have also been used[2,22,25] including the commercially available BOD-SEED consortium.[12]

In the case of the ferricyanide-mediated BOD (FM-BOD) assay, only four species of microorganisms have been reported, although many more have subsequently been investigated in our laboratories. *Escherichia coli* was used as a test organism by both Pasco et al.[14] and Morris et al.[17] to demonstrate the feasibility of the FM-BOD approach. The study by Catterall et al.[16] employed *Pseudomonas putida*, *Bacillus licheniformis*, and *Trichosporon cutaneum* as each was known to have broad range substrate utilization and all have been used in rapid BOD biosensor development.[5] In each of the cases above, the main aim was to demonstrate the feasibility of the FM-BOD approach. Conse-

(30) Reshetilov, A. N.; Efremov, D. A.; Illiasov, P. V.; Boronin, A. M.; Kukushkin, N. I.; Greene, R. V.; Leathers, T. D. *Biosen. Bioelectron.* **1998**, *13* (7−18), 797−799.

(31) Chee, G. J.; Nomura, Y.; Karube, I. *Anal. Chim. Acta* **1999**, *379* (1−2), 185−191.

(32) Morris, K. A ferricyanide mediated approach to rapid biochemical oxygen demand analysis. Honours Thesis, School of Environmental and Applied Science, Griffith University, Gold Coast, 2000.

(33) Catterall, K. Ferricyanide mediated microbial reactions − application to rapid BOD analysis. Honours Thesis, School of Environmental and Applied Science, Griffith University, Gold Coast, 2001.

quently, the FM-BOD assay has, to date, only been applied to artificial samples containing simple organic compounds using a single-species microbial seed. The methodology has yet to be tested using a mixed microbial consortium with real samples.

In this study, we further demonstrate the feasibility of the FM-BOD approach by employing a consortium of microbial species to analyze a range of synthetically prepared organic solutions and real samples. The microorganisms used were *P. putida*, *B. licheniformis*, *T. cutaneum*, and *Proteus vulgaris*. Comparison of the FM-BOD approach with the standard BOD_5 assay was carried out for all samples (artificial and real) tested.

EXPERIMENTAL SECTION

Reagents. All chemicals used in this study were of analytical grade and all solutions were prepared in deionized (Milli-Q water), unless otherwise stated. The BOD[198] standard GGA solution (150 mg L^{-1} glucose/150 mg L^{-1} glutamic acid) was prepared according to procedures described in the APHA standard methods.[27] Potassium ferricyanide (APS) solutions were prepared in phosphate-buffered saline (0.08 M K_2P0_4/0.1 M KCl, pH 7). Standard solutions containing 200 mg L^{-1} each, respectively, of glucose (Chem supply), glutamic acid (BDH), sucrose (Research Organics Inc), glycine (Ajax), starch (Sigma), cellulose (Aldrich), glycerol (Chem Supply), and citric acid (Sigma) were prepared separately in deionized (Milli-Q) water.

Microorganism Preparation. *T. cutaneum*, was a gift from the Adelaide Woman's Hospital, Australia. *P. putida*, *P. vulgaris*, and *B. licheniformis* were purchased from the University of New South Wales Culture Collection facility in Australia. All microorganisms were maintained on nutrient agar plates at 4 °C. Microorganisms were grown aerobically in a shaker bath (75 rpm) for 10 h at 37 °C in Trypticase soy broth (Gibco). A second batch culture was prepared by inoculating Trypticase soy broth with culture (10% v/v) from the previous incubation. Cultures were grown until the end of the exponential phase (approximately 7−8 h) as determined by growth curves. Cells were then harvested by centrifugation at 4500 rpm for 10 min at room temperature, washed twice in phosphate buffer (0.08 M KH_2PO_4/0.12 M K_2HPO_4), and resuspended in phosphate-buffered saline solution (0.08 M KH_2PO_4/0.12 M K_2HPO_4/0.1 M KCl, pH 7). The concentration of cells was adjusted to an absorbance value of 8.0, measured at 600 nm on a diluted solution of cells using a Shimadzu UV-1601 UV− visinle spectrophotometer. The bacterial suspensions were used for the experiments on the day of harvesting.

Sample Preparation and Analysis. Solutions to be incubated typically contained mixtures of microorganisms, ferricyanide, and sample to be analyzed (GGA, organic solutions, or real samples). Each 20.0-mL incubation solution was prepared with the following volumes: 3.3 mL of 0.33 M potassium ferricyanide, 6.7 mL of sample, and 10.0 mL of microorganism solution to give a final microbial absorbance of ∼4.0. In the case of the microbial consortium, 2.5 mL of each of the four microbial suspensions was added. Endogenous control solutions were prepared by replacing the sample solution with deionized (Milli-Q) water. A control blank was also prepared by replacing the microorganisms with phosphate-buffered saline solution (0.08 M KH_2PO_4/0.12 M K_2HPO_4/0.1 M KCl, pH 7).

Samples were incubated at 37 °C in a shaking water bath under oxygen-free nitrogen. One-milliliter aliquots were withdrawn at 30, 60, 90, 120, 150, and 180 min. and the reaction was terminated by centrifugation at 20 000 rpm for 10 min. The supernatant solution was then analyzed for microbially produced ferrocyanide using amperometry at a platinum microelectrode as described previously.[16] The limiting current value obtained for a given sample at a given incubation time was used to determine the ferrocyanide concentration using the equation

$$i_{\mathrm{lim}} = 4nFDrC$$

where i_{lim} is the limiting current (A), n is the number of electrons transferred per mole of ferrocyanide (= 1), F is the Faraday constant (C mol^{-1}), D is the diffusion coefficient (cm^2s^{-1}), r is the radius of the electrode (cm), and C is the concentration of ferrocyanide (mol cm^{-3}).

Calculation of Equivalent FM-BOD Values. Consistent with the BOD_5 assay, the GGA solution was used as a standard check solution to determine microbial seed effectiveness. In this case, amperometric limiting current (i_{lim}) values obtained for a sample throughout the FM-BOD incubation period were divided by the values obtained for the standard GGA solution. This value is termed the "normalized limiting current" and is a unitless parameter indicative of the amount of biodegradation that occurs in a sample relative to the amount of biodegradation that occurs in the standard GGA solution. This parameter was used to take into account any day-to-day (i.e., batch-to-batch) variation in the microbial seeds.

This parameter can be converted to a FM-BOD_5 equivalent value by multiplication by 198 mg/L (the average accepted BOD_5 value for the GGA solution). In all cases, the limiting current values of the endogenous control solution were subtracted from the sample and GGA values prior to the calculation (see below— the term in braces is the normalized limiting current value).

$$\text{FM-BOD}_5 \text{ equivalent value} = \left\{\frac{i_{\mathrm{lim(sample)}} - i_{\mathrm{lim(endogenous)}}}{i_{\mathrm{lim(GGA)}} - i_{\mathrm{lim(endogenous)}}}\right\} \times 198 \text{ mg/L}$$

BOD_5 Analysis. The BOD_5 values for all samples were obtained using the standard method 5210B described by the American Public Health Association (APHA).[27] The microbial seed material used was postgrid filter primary influent collected from a local wastewater treatment plant. After a 200-fold dilution, the seed material was prepared as described by APHA.[27]

Correlation Analysis. Where valid, the Pearson correlation coefficient was used as a measure of the intensity of association between the values obtained from the FM-BOD method and the BOD_5 method. This was employed for the data in Figure 3. Where the data was not normally distributed (an assumption of the Pearson product moment) the nonparametric Spearman ρ correlation was used. This was employed for the data in Table 1.

To characterize the trend of the two-way scattergram in Figure 3, the slope of the principal axis of the correlation ellipse was calculated. This was done in preference to simple linear regression analysis since regression analysis assumes the x values are measured with no (or negligible) error and that the y values are the dependent, normally distributed variables. This is not the case for correlation between FM-BOD values and BOD_5 values.

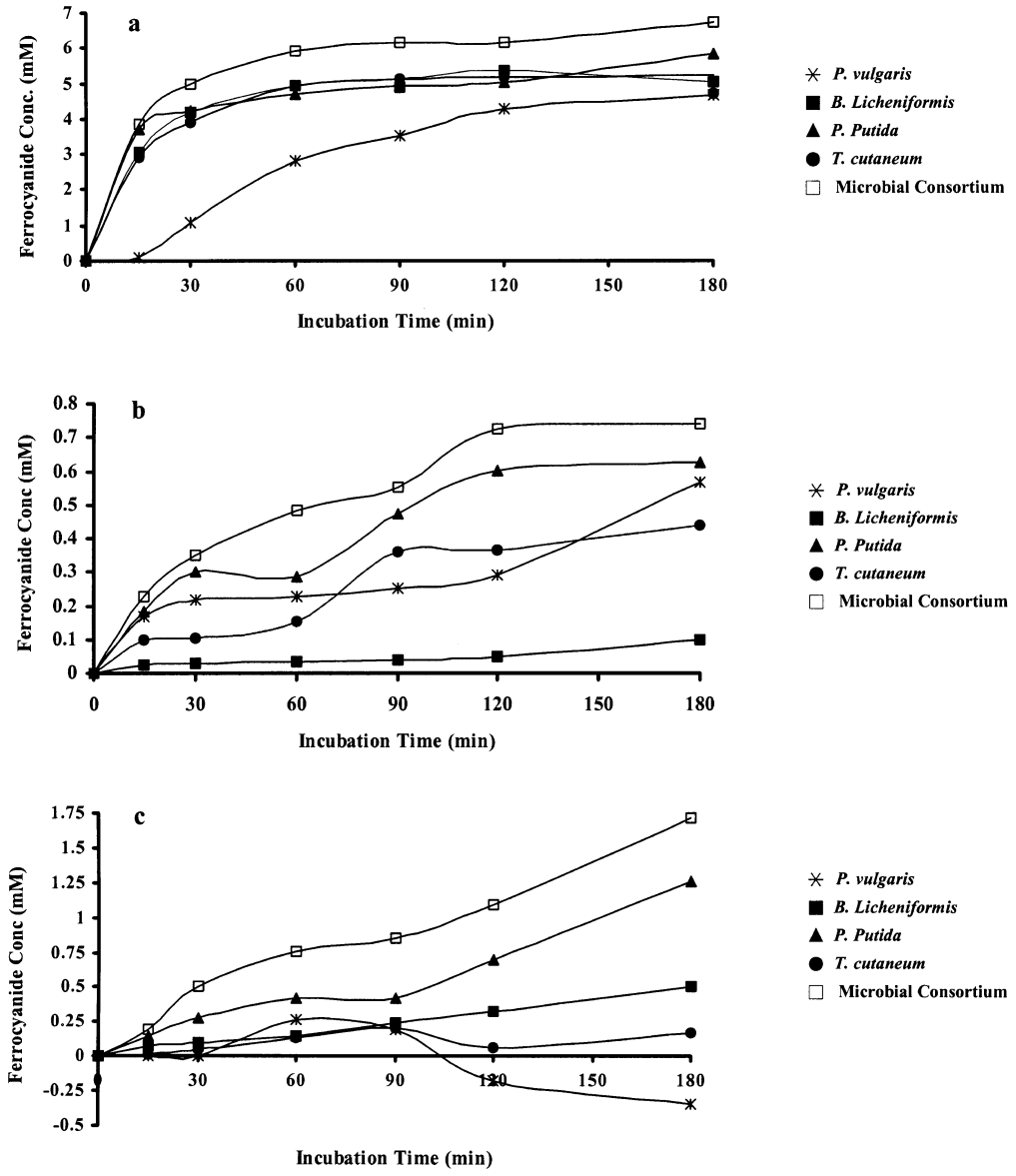

Figure 1. Ferrocyanide concentration obtained at various incubation times for individual microbial species and the mixed microbial consortium. All sample solutions were incubated in the presence of the standard GGA solution (a) or food process samples (b, c). Endogenous control values have been subtracted. Final microbial absorbance, 4; ferricyanide final concentration, 55 mM. Limiting currents determined by chronoamperometry at E_{app} = +450 mV (vs Ag/AgCl).

Real Samples. Real samples were obtained from the following locations: biscuit factory (×3), cake factory (×2), cannery (×4), brewery (×2), sugar mill (×2), pond water (×2), carwash, pet food factory, butcher (×2), bakery, wastewater treatment plant (primary influent), domestic (×7), cafeteria, and dairy factory. FM-BOD and BOD_5 analyses were performed as described above in Sample Preparation and Analysis and BOD_5 Analysis, respectively.

RESULTS AND DISCUSSION

Comparison of Single-Species and Multispecies Microbial Seed in the FM-BOD Assay. A comparison of the ability of the single species and the synthetically prepared microbial consortium to rapidly and fully degrade organic solutions was made. Figure 1a shows how the ferrocyanide concentration (indicative of the extent of the biochemical reaction) varied over a 3-h period for the four microbial seeds and the microbial consortium in the standard GGA solution. With the exception of *P. vulgaris*, the ferrocyanide concentration obtained for each seed increased rapidly, within the first 15 min. The mixed consortium seed, in particular, was very rapid and resulted in the highest ferrocyanide concentration throughout the incubation time investigated. Bearing in mind that the final absorbance values of each seed were identical, this result implies that the combination of microbes had a synergistic effect on catabolism of the organic sample. This phenomenon was likely to be due to the microbes feeding off the nonfully degraded byproducts of the other species. When incubated individually, *P. vulgaris*, resulted in the lowest ferrocyanide production, indicating a relatively limited capacity to rapidly and fully degrade the GGA solution. However, in the presence of other microbial species, the byproducts produced by these species may have provided a more readily biodegradable food source. Conse-

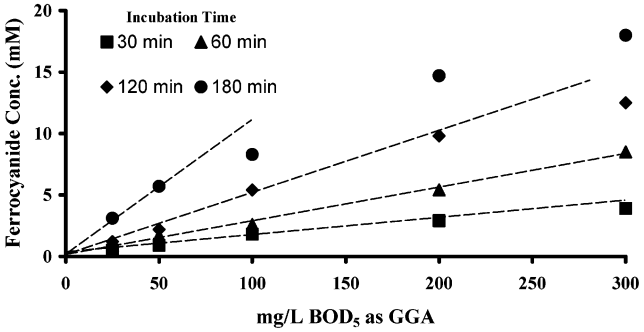

Figure 2. Determination of the dynamic linear range for the mixed microbial consortium FM-BOD using GGA as the test organic substrate. Endogenous control values have been subtracted. Microbial consortium final absorbance 4; ferricyanide final concentration, 55 mM. Limiting currents determined by chronoamperometry at E_{app} = +450 mV (vs Ag/AgCl).

quently, an overall increase in the biochemical reaction for the mixed consortium incubation would result. This scenario is likely to be true for each of the other microbes within the multispecies incubation mixture.

The improved capacity of the mixed consortium to degrade organic solutions in the presence of ferricyanide was generally more pronounced with real sample effluents than with synthetically prepared organic standards. Panels b and c of Figure 1 show how ferrocyanide production varied over the 3-h incubation period for two food process effluents. In both cases, the rate and extent of reaction was significantly improved for the mixed consortium relative to the individual species, indicating that a greater extent of biodegradation/biooxidation had occurred. In the example shown in Figure 1b, this occurred despite the fact that *B. licheniformis* did not degrade the sample effluent to any appreciable extent. The high variability of response (over time) for the single-species seeds was also evident in both cases. Again, the mixed consortium responses were not only larger but less fluctuating over time. Of particular interest in Figure 1c were the negative responses obtained for *P. vulgaris* at the 2- and 3-h incubation times. These negative values were the result of the endogenous control values being higher than the sample values and indicated an inhibitory effect of some kind. Despite this, the mixed consortium still showed appreciably higher degradation efficiencies, even at 2 and 3 h.

While the mixed consortium showed increased rates and extent of biodegradation, no significant trends were observed for the relative performance of the individual species. The responses derived from *P. putida*, however, were prominent in all three analyses shown and tended to be in most other sample solutions as well. This was consistent with our previous study,[16] which showed that *P. putida* was the most promising of the four species used in that study.

Linear Dynamic Range. The performance of the mixed microbial consortium in FM-BOD analysis was further investigated using the GGA standard solution to determine the linear dynamic range of the method. Figure 2 shows the effect of varying the concentration of GGA (up to 300 mg BOD_5 L^{-1}) at each incubation time using the mixed microbial consortium. Reasonable linearity below 200−300 mg BOD_5 L^{-1} was observed for the 30-, 60-, and 120-min incubation times. This was similar to the linear ranges reported in previous FM-BOD studies employing different microbial seeds.[16,32] Deviation from linearity above 50 mg BOD_5 L^{-1} was observed for the 3-h incubation time. These results imply that if a real sample were to undergo biodegradation at rates similar to the GGA sample, then dilution to below 200−300 mg BOD_5 L^{-1} would be required for incubation times of 2 h or less. In the case of a 3-h incubation, dilution to below 50 mg BOD_5 L^{-1} would be required.

Table 1. Correlation Coefficients Obtained by Comparison of the FM-BOD Assay with the BOD_5 for the Five Microbial Seeds Using Synthetically Prepared Organic Solution and Real Samples

	Spearman ρ correlation coefficient (p value)		
microbial seed	organic ($n = 8$)	real ($n = 9$)	combined ($n = 17$)
B. licheniformis	0.405 (0.320)	0.033 (0.932)	0.213 (0.411)
T. cutaneum	0.524 (0.183)	−0.200 (0.606)	0.103 (0.694)
P. vulgaris	0.548 (0.160)	0.417 (0.265)	0.437 (0.079)
P. putida	0.929 (0.001)[a]	0.433 (0.244)	0.475 (0.054)
mixed consortium	0.905 (0.002)[a]	0.983 (0.000)[a]	0.873 (0.000)[a]

[a] Significant at the 0.01 level (two-tailed).

Importantly, the linear ranges demonstrated in Figure 2 are much greater than for the standard BOD_5 method (approximately 1−8 mg/L BOD_5) and most BOD biosensors reported (see ref 24 and references therein). In the case of the BOD_5 assay, the linear range is limited by the availability of poorly soluble O_2. In the case of BOD biosensors, diffusional limitations across the immobilized biocatalytic layer allow linear ranges to reach (or sometimes exceed) 50 mg BOD_5 L^{-1}. In our method, the high concentrations of both biocatalyst and electron acceptor mean that, typically, the organic substrate is the limiting reactant. Only at high substrate concentrations do the biocatalyst or electron acceptor limit the reaction.

FM-BOD Analysis of Organic Standards and Real Samples. Correlation to BOD_5. The individual microbial seeds and the mixed consortium were further compared in their ability to estimate the BOD_5 values of synthetically prepared organic solutions ($n = 8$) and a collection of real industrial/domestic waste effluents ($n = 9$). The linear dynamic range of the method necessitated the dilution of real samples to within the range 0−200 mg BOD_5 L^{-1}. The standard organic solutions were also prepared within this range. BOD_5 values and equivalent FM-BOD_5 values were determined for all 17 solutions—the latter employing both individual microbial seeds and the mixed consortium.

Table 1 shows the correlation information between the FM-BOD method and the BOD_5 method for the 17 samples for all 5 microbial seed preparations. Also shown are correlation coefficients (and p values) for the separate organic and real samples solutions. Lack of normality in the data, coupled to a relatively small data set, necessitated the use of the Spearman ρ analysis in order to statistically test the correlation between the two methods.

In general, poor correlation between the two BOD methods was found when a single species microbial seed was employed in the FM-BOD assay. This was confirmed by poor correlation coefficients (R was <0.5 in all four combined cases) and a lack of statistical significance of correlation at the $p \leq 0.05$ level. Interestingly, the data for the *P. putida* seed was quite good for the synthetic samples but not so for the real samples. By contrast,

the results for the mixed microbial consortium showed a highly significant correlation between the two methods in all three scenarios with $R = 0.873$ and $p = 0.000$ for the 17 combined samples. This marked improvement between the two methods shows the promise of the FM-BOD approach for rapid BOD analysis when a mixed microbial consortium is employed. It also indicates that the nature and extent of the degradation observed in the rapid FM-BOD process (using the microbial consortium described) more closely represented the nature and extent of degradation found in the BOD_5 method. In one respect, this is not surprising since the BOD_5 method also employs a microbial consortium. The fact that we see similarity between the methods is noteworthy, especially given that one employs ferricyanide-mediated catabolism and the other conventional aerobic catabolism.

The success of the microbial consortium in FM-BOD analysis, in terms of both increased rate and extent of the biochemical reaction and strong correlation with the BOD_5 assay, prompted further testing with an increased number and a wider diversity of real samples. The samples chosen represent a diverse range of industrial/domestic waste effluents in which BOD_5 assays are typically employed (see Real Samples). The samples also varied greatly in their biodegradability with BOD_5/COD ratios ranging from 0.015 (poorly biodegradable) for the treated cannery effluents up to 1.03 (very biodegradable) for the sugar mill effluent. Other BOD_5/COD values include 0.05 for treated brewery effluent, 0.13 for treated sugar pond effluent, 0.39 for the dairy effluent. and 0.41 for the biscuit factory effluent.

Figure 3 shows the correlation between the FM-BOD method and the BOD_5 method for the 30 real samples obtained from the industry/domestic process and wastewater sources listed in the Experimental Section. Figure 3a is a plot of the average normalized limiting current values obtained throughout the 3-h FM-BOD incubation versus the measured BOD_5 values. A highly significant correlation ($R = 0.935$, $p = 0.000$, $n = 30$) between the two methods was observed. Figure 3b shows the same data converted to FM-BOD_5 equivalent values. This was done in an effort to equate the two methods in terms of measurable BOD quantities—after all, both methods indirectly measure the same parameter, i.e., the amount of electron transfer to a terminal electron acceptor. In making this conversion, the GGA solution is now effectively being used as a calibration standard—rather than a standard check solution to check day-to-day seed integrity. The use of GGA as a calibration standard has been shown previously in the rapid BOD biosensor approach,[5,7,25,26] although the appropriateness of doing so has been raised by several authors.[13,15,16] Because of the relatively high biodegradability of GGA, it has been questioned whether this solution can be employed as a good calibration standard across a wide diversity of sample types. This is despite the fact that the rate of biodegradation of GGA "is similar to many municipal wastes".[27] Recent rapid BOD studies (e.g., ref 19) have employed the OECD wastewater standard as the calibration standard. While, good correlation was obtained for samples of the same type, different slopes were obtained for different sample types, indicating that correlation across different sample types was poor (see ref 19).

In the present work, however, the use of GGA as the calibration standard for FM-BOD analysis seemed to work well for most of the sample types investigated. This was evidenced by the high correlation coefficient and the fact that the slope of the principal axis of the correlation ellipse in Figure 3b was found to be close to unity (i.e., 0.92). A slope of 1 would be expected if both methods were accurately measuring the same BOD value. While most of the data points lie below the ideal (slope of 1) line, the 95% confidence interval of this slope was 0.80−1.05, which means that we can be 95% confident that the true slope lies just between these two values. Given that there are analytical errors associated with both the BOD_5 and FM-BOD measurements and that these errors contribute to scatter on both axes, the strong correlation (and slope) obtained provides compelling support for the suitability of the FM-BOD method for measuring biochemical oxygen demand—particularly given the large number and wide diversity of samples analyzed.

The fact that a significant correlation was achieved for such a wide diversity of real samples demonstrates that the microbial consortium used in the FM-BOD assay is capable of rapidly degrading a wide range of organic substrates in complex matrixes in much the same way as the microbial seed in the conventional 5-day BOD_5 assay. Comparison of the extent of biochemical biooxidation of the 30 real samples by both methods confirms this. It was found that the relative extent of oxidation observed for the 3-h FM-BOD incubation varied between 26 and 125% of the amount of biooxidation observed for the 5-day BOD_5 incubation. The average value for the 30 samples was 63 ± 12%. These values were calculated by comparing the amount of electrons that were biochemically transferred to ferricyanide in the 3-h FM-BOD

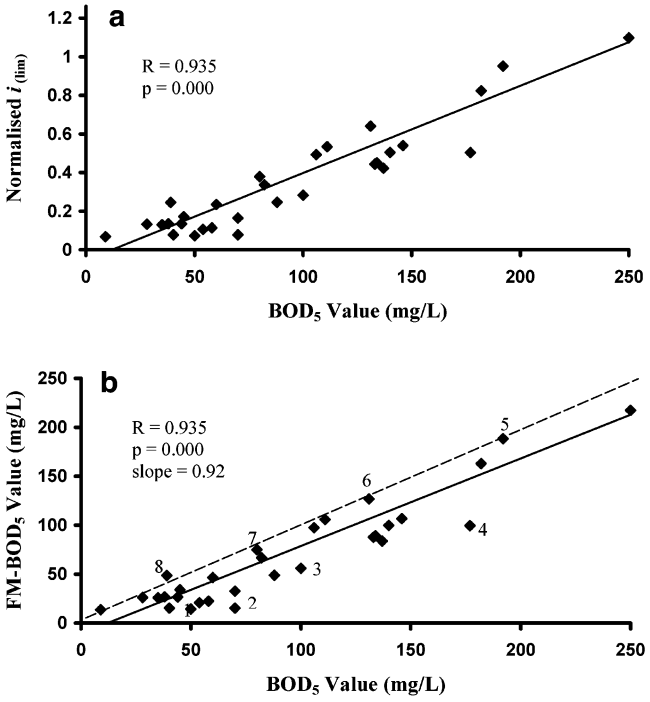

Figure 3. (a) Scatterplot showing the relationship between the normalized limiting current values (obtained by the FM-BOD method) and the BOD_5 values for 30 real samples using the mixed microbial consortium. Also shown is the line representing the principal axis of the correlation ellipse. (b) Scatterplot of FM-BOD_5 equivalent values versus BOD_5 values for the data shown in (a). The solid line is the principal axis of the correlation ellipse; the dotted line is the ideal slope, = 1 line.

process (by measuring ferrocyanide increase) with the amount of electrons biochemically transferred to O_2 in the BOD_5 assay (by measuring oxygen depletion). It is worth noting that, on average, 63% of the biooxidation that occurred in the 5-day BOD_5 assay occurred in 3 h for the FM-BOD assay. This is considerably higher than any other biological-based rapid alternative for BOD analysis reported to date.

Closer evaluation of the data in Figure 3 reveals an interesting trend with respect to the strength of the correlation of the two methods. For example, the FM-BOD_5 equivalent values for the outliers in Figure 3b (numbered 1−4) were shown to significantly underestimate the BOD_5 values. These samples were the pet food factory effluent (1), wasterwater treatment plant primary influent (2), dairy effluent (3), and biscuit factory effluent (4). The amount of biooxidation that occurred in the FM-BOD incubation period relative to the 5-day BOD_5 assay was only 26, 32, 42, and 34, respectively—well below the average of the other samples. Conversely, the corresponding values for samples that correlate very well were much higher. For example, the raw cannery effluent (5), pond water (6), domestic effluent (7), and cafeteria effluent (8) had relative biooxidation values of 61, 103, 67, and 60%, respectively. It seems, therefore, that two criteria need to be satisfied to achieve reasonable correlation between the two methods. The first is that the sample needs to be diluted below ~200 mg BOD_5 L^{-1}. The second is that the amount of biooxidation of the sample during the FM-BOD incubation period needs to be relatively high relative to the BOD_5 assay. The high microbial population and diversity of species in our microbial consortium allow this to be achieved within 3 h for most of the samples investigated in this study. This may also explain why correlation to the BOD_5 assay across different sample types is difficult for BOD biosensors. This is because (i) the short assay time results in minimal degradation (compared to the BOD_5 assay) and (ii) the use of microbial seeds with minimal species diversity results in highly variable catabolic efficiencies for different sample types—especially when compared to the consortium employed in a BOD_5 assay. Given these limitations, one would not expect correlation between a BOD biosensor and the BOD_5 assay across a wide range of sample types.

CONCLUSIONS

While a rapid alternative to the BOD_5 assay will never exactly duplicate the microbial processes and results obtained in a true BOD_5 assay, the need to come up with viable rapid alternatives still remains. Many of the experimental conditions under which the BOD_5 assay is employed (e.g., the 5-day duration) are arbitrary and are only still in place for historical reasons. Viable, faster, alternatives to these procedures with comparable biodegradation efficiencies that give quantitatively similar results should be seriously considered. The FM-BOD approach outlined in this study has achieved this for a large number and wide diversity of industrial/domestic effluents.

This study has also shown that employing a multispecies microbial seed in the FM-BOD assay provides significant advantages over the use of a single-species microbial seed. In particular, increased rates of biodegradation and markedly improved correlation to the standard BOD_5 assay were noted. An increase in linear dynamic range (decreasing dilution requirements) and considerably higher biodegradation efficiencies, compared to other rapid BOD bioassays, were also noted. These advantages, coupled to a highly significant correlation ($R = 0.935$, $p = 0.000$, $n = 30$) between the FM-BOD assay and the BOD_5 assay for a wide diversity and large number of real samples, indicate that this new assay shows real promise as a rapid alternative to the BOD_5 assay.

As with any rapid BOD method, the effectiveness of the microbial seed is paramount to the eventual performance of the assay. Current work is centered on investigations of other microbial species (and combinations) and on the preparation of an immobilized seed suitable for long-term storage and/or reuse.

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