A ferricyanide-mediated activated sludge bioassay for fast determination of the biochemical oxygen demand of wastewaters

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Abstract
Activated sludge was successfully incorporated as the biocatalyst in the fast, ferricyanide-mediated biochemical oxygen demand (FM-BOD) bioassay. Sludge preparation procedures were optimized for three potential biocatalysts; aeration basin mixed liquor, aerobic digester sludge and return activated sludge. Following a 24 h starving period, the return activated sludge and mixed liquor sludges reported the highest oxidative degradation of a standard glucose/glutamic acid (GGA) mixture and the return activated sludge also recorded the lowest endogenous FM-respiration rate. Dynamic working ranges up to 170 mg BOD5 L⁻¹ for OECD standard solutions and 300 mg BOD5 L⁻¹ for GGA were obtained. This is a considerable improvement upon the BOD5 standard assay and most other rapid BOD techniques. Time-series ferricyanide-mediated oxidation of the OECD170 standard approached that of the GGA198 standard after 3–6 h. This is noteworthy given the OECD standard is formulated as a synthetic sewage analogue. A highly significant correlation with the BOD5 standard method (n = 35, p < 0.001, R = 0.952) was observed for a wide diversity of real wastewater samples. The mean degradation efficiency was indistinguishable from that observed for the BOD5 assay. These results demonstrate that the activated sludge FM-BOD assay may be used for simple, same-day BOD analysis of wastewaters.

1. Introduction
Developed in 1936, the 5-day biochemical oxygen demand (BOD₅) assay remains the only internationally recognized standard method for estimating the biodegradable organic loading of wastewaters (APHA, 1999). The BOD₅ bioassay is a direct estimate of the amount of oxygen consumed by microbial respiration under standard incubation conditions. Most determinations of BOD₅ are made on samples of wastewater or treated effluent to meet regulatory requirements (Higgins et al. 2004). The assay commonly incorporates a mixed microbial seed activated sludge from a wastewater treatment plant (WWTP) as the biocatalyst. The incorporation of a biological component in the assay means that BOD₅ measurements can be used to provide an estimate of the biodegradability and hence the oxygen demand of a sample.
This is a significant advantage of the assay compared to rapid surrogates such as chemical oxygen demand (COD) measurements, which cannot differentiate between the biodegradable and non-biodegradable fraction of organics (Zhao et al., 2004). However, due to the low solubility of oxygen in water (8.7 mg O₂ L⁻¹ at 25 °C) and the limitations of dissolved oxygen electrodes, BOD₅ measurements must be measured over multiple serial dilutions to ensure that changes in dissolved oxygen concentrations are able to be accurately quantified (APHA, 1999). The requirement for the assay to extend over 5 days is partly to ensure sufficient degradation of the waste material, 60.5% substrate oxidation is typical (APHA, 1999), and partly an historical idiosyncrasy (Baird and Smith, 2002). However, this duration also represents the most significant shortcoming of the assay, as it would not be uncommon for the results of assays to be obtained long after the wastewater or effluent being measured had to be released.

Given the need to decrease analysis time and the costs of undertaking BOD₅ analysis, BOD biosensors were developed (Karube et al., 1977) and have attracted considerable interest (D’Souza, 2001; Liu and Mattiasson, 2002; Rodriguez-Mozaz et al., 2005). BOD biosensors measure the bacterial respiration rate, at an oxygen electrode interface, as a function of biodegradable material in as little as 1–20 min (Liu and Mattiasson, 2002). However, as the analytical signal consists of monitoring the flux of oxygen to the electrode surface, BOD biosensors are also constrained by oxygen solubility. The biological component generally consists of an immobilized aerobic bacterial population (often grown as a biofilm) with a broad substrate range or preferably a consortium of heterotrophs (D’Souza, 2001; Liu and Mattiasson, 2002). Another issue with BOD biosensors is that generally <1% of available biodegradable organics are mineralized over the duration of the measurement (Morris et al., 2001). This means the most readily degradable substrate pools are over-represented in the analysis, which can lead to overestimation of the BOD of a sample. A small number of studies have sought to partially overcome this inherent limitation by successfully immobilizing activated sludge as the biocomponent (Liu et al., 2000; Matsuoka and Isoda, 2005; Sakai et al., 1995). However, practical application of the biosensors is limited, given the instability of the community composition over time and their tendency for biofouling (Liu and Mattiasson, 2002).

In view of the intrinsic problems associated with utilizing oxygen as the terminal electron acceptor, Yoshida and co-workers developed a BOD biosensor replacing oxygen with a reox mediator, the ferricyanide ion (Yoshida et al., 2001, 2000). Results gained using this novel approach compared very favorably with existing oxygen-based biosensors, but still suffered from the limitations common to biosensors. Around very favorably with existing oxygen-based biosensors, but still suffered from the limitations common to biosensors. Around very favorably with existing oxygen-based biosensors, but still suffered from the limitations common to biosensors.

Ferricyanide-mediated biodegradation proceeds via glycolysis and the tricarboxylic acid cycle (Morris et al., 2005) and is analogous to aerobic respiration (Eq. (1a)). However, in the absence of oxygen, the electrons produced via substrate oxidation are shuttled down the electron transport system to reduce the terminal electron acceptor ferricyanide to ferrocyanide (Eq. (1b)). The overall process of ferricyanide-mediated (FM) respiration is displayed in Eq. (1c) (Morris et al., 2005).

\[
\text{CH}_2\text{O} + \text{H}_2\text{O} \xrightarrow{\text{Glycolysis/TCA}} \text{CO}_2 + 4\text{H}^+ + 4e^- \\
[\text{Fe(CN)}_6]^{3-} + 4e^- \xrightarrow{\text{ETS}} 4[\text{Fe(CN)}_6]^{4-} \\
\text{CH}_2\text{O} + \text{H}_2\text{O} + 4[\text{Fe(CN)}_6]^{3-} \xrightarrow{\text{Biocatalyst}} \text{CO}_2 + 4\text{H}^+ + 4[\text{Fe(CN)}_6]^{4-}
\]

Since its inception in 2000, considerable research into commercialization and optimization of the FM-BOD method and biocatalyst has been undertaken (Catterall et al., 2001, 2003; Morris, 2005; Morris et al., 2001, 2003; Pasco et al., 2004). Early studies employing Escherichia coli generated a great deal of interest in the FM-BOD assay as reported glucose-glutamic acid (GGA) degradation efficiencies were comparable with the BOD₅ standard method and yet the total analysis time was a little over an hour (Morris et al., 2001; Pasco et al., 2000). Achieving degradation efficiencies of this magnitude over such a short space of time is possible by virtue of the high solubility of ferricyanide in water compared with oxygen. Ferricyanide is ~10,000 fold more soluble in water than O₂ and consequently a considerably larger population of microorganisms may be used as the biocomponent without significant depletion of the electron acceptor (i.e. ferricyanide). Unlike the BOD₅ assay and oxygen-based biosensors, which are limited by the electron acceptor concentration, the rate-limiting reactant in the FM-BOD assay is the substrate, which is characteristically consumed according to the Michaelis-Menten model (Lehninger, 1970). Thus, the reaction rate is quantitatively related to the substrate concentration.

The choice of biocatalyst in any bioassay is of critical importance. E. coli represented a good biocatalyst for the early proof of principle studies (Morris et al., 2001; Pasco et al., 2000) chiefly because ferricyanide-mediated respiration is well characterised for the species (Emde et al., 1989; Patchett et al., 1989). In particular, an important recent study (Catterall et al., 2010) has clearly demonstrated that FM-respiration responds quantitatively to both stimulation and inhibition of E. coli activity, validating the analytical technique for the first time and confirming its suitability as a BOD assay. Nevertheless, in spite of excellent degradation efficiencies over short time scales, E. coli is not recognized as a suitable species for rapid BOD analysis, given its limited ability to assimilate a wide range of organic substrates. In order to extend the substrate spectrum, a defined consortium of pure culture microorganisms from diverse heterotrophic groups was employed as the FM-BOD biocatalyst (Catterall et al., 2003; Morris et al., 2003). Using the GGA standard as a calibrating solution, excellent agreement was observed between FM-BOD and BOD₅ measurements for a large number of industrial effluent samples (n = 27–30) in as little as three hours. However, in some cases relatively recalcitrant samples were grossly underestimated (Catterall et al., 2003; Morris et al., 2003).

It became apparent that if the FM-BOD assay was ever to be considered for widespread and facile BOD determination of industrial wastewater samples, varying greatly in terms of...
BOD₅ value and biodegradability, a more diverse biocatalyst was needed. Successful integration of activated sludge as the biocomponent in an FM-BOD assay would overcome the time-consuming and expensive requirement of culturing several strains of bacteria to produce a mixed consortia biocatalyst. Activated sludge also represents the ideal biocatalyst for the BOD₅ assay as it contains a diverse community of heterotrophs from a number of functional groups that will ensure a high proportion of biodegradation occurs (Dias and Bhat, 1964, 1965; Hiraishi et al., 1989). In addition, activated sludge can be readily sampled from most WWTPs and an activated sludge FM-BOD assay would be far more relevant to the wastewater industry, as measurements of the industrial effluent BODs, would be representative of the BOD loading exerted upon the plant activated sludge ultimately treating the same wastewater.

The primary objective of this study was therefore to develop an FM-BOD assay utilizing activated sludge as the biocatalyst. A number of experimental conditions were optimized with regards to increasing net FM-respiration of the sludge. The rate and pattern of substrate degradation over time was investigated using both the GGA¹⁹⁸ and OECD¹⁷⁰ standard solutions. These standards were also used for calibration of the assay and determining the working range, which is based upon adherence to the Michaelis-Menten model. Finally, the utility of the assay for rapidly predicting BOD₅ values was investigated by correlation with the BOD₅ assay, using a range of real industrial effluents of varying complexity and BOD₅ value.

2. Experimental

2.1. Reagents

All reagents used in this study were of analytical reagent grade and all dilutions were made using deionized (Milli-Q, Element) water, unless otherwise stated. The BOD¹⁹⁸ GGA and BOD¹⁷⁰ OECD synthetic wastewater standard solutions were prepared according to the APHA standard methods (APHA, 1999) and OECD-209, activated sludge respiration inhibition assay (following a 100-fold dilution) (OECD-209, 1984) respectively. Potassium ferricyanide (Ajax) solutions were prepared in phosphate buffer (PB) (0.08 M KH₂PO₄/0.12 M K₂HPO₄, pH 7, Chem-supply).

2.2. Activated sludge preparation

The type of sludge used in the assay, the absorbance of the sludge and the starvation period were optimized in this study. Further details on the sludge characteristics are in the supporting information (Table S-1) and in Section 3.1. All sludge was collected fresh as required from Coombabah WWTP, Queensland, Australia. In the laboratory, the sludge was vigorously shaken with glass beads for ~1 min, wet sieved through a 500 µm sieve with PB and washed, centrifuged (1 min at 1300 rcf) and resuspended in PB. This step was repeated once. The final sludge/PB slurry was then aerobically incubated on an orbital shaker (100 rpm) at room temperature for the stated starvation period. The sludge microorganisms were then harvested by centrifugation and the absorbance of a diluted suspension of sludge was determined spectrophotometrically at 600 nm. The sludge was then diluted to the stated absorbance (optical density) using PB.

2.3. Sample preparation and analysis

Solutions to be incubated contained mixtures of activated sludge, ferricyanide and the sample/standard to be analyzed. Each 6.0 mL incubation solution was prepared with the following volumes: 3.0 mL of activated sludge, 2.0 mL of sample/standard and 1.0 mL of 0.36 M potassium ferricyanide (final concentration 0.06 M). Controls to determine endogenous metabolism (baseline metabolic rate in the absence of added substrate) were prepared by replacing the sample/standard with Milli-Q water and sample interference blanks were prepared by substituting the activated sludge with a 10 mM potassium ferrocyanide/ferricyanide solution in PB.

The optimal incubation time of the assay will depend upon the nature of the samples to be analyzed. Further details are provided in the results and discussion section. Samples were incubated in 10 mL plastic vials at 20 ± 1 °C (consistent with the BOD₅ assay) on an orbital shaker. Approximately 1.5 mL aliquots were subsampled at 3 and 6 h into 2 mL microtubes and the reaction was terminated by centrifugation at 13,700 rcf for 3 min (Catterall et al., 2003). The supernatant was analyzed for ferrocyanide using chronoamperometry at a 25 μm platinum working electrode, as described previously (Catterall et al., 2001; Morris et al., 2001). The analytical signal, the diffusion limiting current, is proportional to the flux of ferrocyanide to the electrode interface (Eq. (2)) and is therefore proportional to the concentration of ferrocyanide in the bulk solution, according to the equation (Catterall et al., 2003):

\[ i_{\text{lim}} = 4nF\pi DC \]  

(2)

Where \( i_{\text{lim}} \) = the limiting current (A); \( n \) = the number of electrons transferred per mole of ferrocyanide (= 1); \( F \) = the Faraday constant (C mol⁻¹); \( r \) = the microelectrode radius (cm); \( D \) = the diffusion coefficient of ferrocyanide (cm² mol⁻¹ s⁻¹) and; \( C \) = the concentration of ferrocyanide in the bulk solution (mol cm⁻³).

2.4. Calculation of FM-BOD equivalent values

As with the BOD₅ assay, either the GGA¹⁹⁸ or OECD¹⁷⁰ standard solutions were used as a standard check to determine microbial seed effectiveness, or in this case, standardize sludge activity. Calculation of FM-BOD equivalent values for real samples relies upon a four-point calibration curve that is based upon adherence to the Michaelis-Menten model (Eq. (3)). The four calibration standards (three standards plus one blank) are incubated and analyzed alongside real samples. Therefore, amperometric limiting current (\( i_{\text{lim}} \)) values derived from a real sample can be easily converted to an equivalent FM-BOD value, relative to either of the BOD₅ standard solutions, once the coefficients \( a \) and \( b \) are empirically defined (Eq. (3)). In all cases, the limiting current for the control endogenous metabolism incubation was subtracted from the
sample/standard gross limiting current value prior to calculation of FM-BOD values.

\[
[\text{FM} - \text{BOD}] = \frac{b \cdot i_{\text{lim}}}{a - i_{\text{lim}}}
\]  

(3)

Where \( i_{\text{lim}} \) = the corrected analytical signal (sample – endogenous control) and represents the stimulation of the rate of the enzymatic reaction; \( a \) = the maximum reaction rate and; \( b \) = the Michaelis-Menten rate constant (Lehninger, 1970).

2.5. BOD\textsubscript{5} and statistical analysis

BOD\textsubscript{5} values were derived according to the APHA 5210B standard method (APHA, 1999). The microbial seed used for the assay was return activated sludge (RAS) from a local WWTP (Coombabah, Gold Coast Water). Following a 3000-fold dilution, the seed was prepared as described (APHA, 1999). The slope of the principal axis of the correlation ellipse was determined as described previously (Catterall et al., 2003; Morris et al., 2003). All assumptions were met and subsequently the data was not transformed.

2.6. FM-BOD limit of detection

Ten separate samples, each containing 10 mg BOD\textsubscript{5} L\textsuperscript{-1} OECD standard solution were incubated for 3 and 6 h and analyzed for microbiologically produced ferrocyanide, less the endogenous contribution. The method detection limit (3 \( \times \) S of the limiting current values) was converted to an FM-BOD detection limit concentration as described above (Eq. (3)) and equaled 13.7 and 9.78 mg FM-BOD L\textsuperscript{-1} for the 3 and 6 h incubations, respectively.

3. Results and discussion

3.1. Biocatalyst optimization

Return activated sludge, aerobic digester activated sludge (ADS) and aeration basin mixed liquor (ML) were trialed as possible biocatalysts for use in the rapid FM-BOD assay, using the GGA\textsuperscript{198} standard as the sample. Preliminary assessments revealed that, with no starvation period prior to sludge washing and incubation, the contribution of endogenous metabolism to total ferrocyanide production for all sludges was unacceptably high, at \( \approx 80\% \), thus decreasing the sensitivity of the assay (Fig. 1); treatment (a). Two treatments were investigated to reduce this effect, both incorporating a 24 h starvation period prior to incubation, but with the sludge either washed after (b) or before starving (c). Fig. 1 shows that the contribution of endogenous respiration to the analytical signal was reduced most effectively by washing the sludge prior to the 24 h starving period (treatment c), with the endogenous contribution representing 64, 69 and 67\% of the total for the RAS, ADS and ML sludges respectively. This proportion is still relatively high compared to those reported for pure culture microorganisms (Catterall et al., 2001; Morris et al., 2003; Pasco et al., 2004), although not unexpected. In addition to living microorganisms, the flocs present in activated sewage sludges contain particulate and soluble organic matter derived from the wastewater, microbiobically produced polysaccharides and dead cells (Bura et al., 1998), all of which could fuel respiration in the absence of exogenously added organic carbon. In addition to the improved endogenous response, treatment c also recorded the highest net FM-respiration rate, i.e. GGA substrate oxidation only, representing an increase of 242\% for the RAS, 180\% for the ADS and 162\% for the ML compared with treatment a (Fig. 1), thereby greatly improving the sensitivity of the assay. This disparity between sludges is influenced, at least in part, by the relative population density of heterotrophs in each sludge and their metabolic state. The aeration basins, where the ML and ultimately the RAS are sampled from, select for predominantly heterotrophic bacteria, while the ADS sludge consists of both heterotrophs and autotrophic nitrifying bacteria, selecting for the latter due to carbon limitations.

A follow-up study investigated the optimal starvation period for each sludge (i.e. 12, 24, 48 and 72 h). 24 h represented the best compromise between maximizing sensitivity and minimizing both the endogenous response and time constraints, which would limit WWTP applications (Fig. S-1). Results gained from both optimization studies suggest that the RAS and ML have a superior net sensitivity and a lower endogenous contribution than the ADS. The RAS was chosen as the biocatalyst for all subsequent FM-BOD incubations, in part due to the lower endogenous respiration, but also the RAS is maintained at a total suspended solids concentration 3 fold that of the ML (Table S-1) and therefore in practical terms, the volume of RAS requiring centrifugation/washing is a third that of the ML.
3.2. Microorganism concentration

The optical density of the RAS was optimized with regard to maximizing sensitivity and the dynamic working range of the method and yet maintaining a sludge density that could be easily prepared. Three replicate incubations investigated FM-respiration at 12 different sludge absorbances ranging from 1−15 (final concentration). Mean net ferrocyanide production remained linear up to optical density (OD) 13 (n = 10, R² = 0.997, p < 0.001), at which point the additional ferrocyanide production started to decrease with increasing OD. However, beyond OD 10 the sludge was too thick to be quantitatively pipetted and instead required individual weighing into each sample tube. Moreover, a choice of OD 10 required no compromise between sensitivity and the dynamic working range of the assay (data not shown). Thus, a final OD of 10 was chosen for all subsequent incubations.

3.3. Time-series substrate oxidation

The pattern of substrate degradation over time was investigated using the GGA198 and OECD170 standard solutions (Fig. 2). The net rate of GGA oxidation surpassed that observed for the OECD standard over the first three incubation hours. This was not unexpected given the higher BOD5 value of the GGA standard over the first three incubation hours. This was investigated in the following two sections.

Fig. 2 – Time-series oxidation of the GGA198 and OECD170 standard solutions as measured by ferrocyanide production. Each point represents the mean response from three replicate incubations. Endogenous control values have been subtracted. RAS was used as the biocatalyst; final sludge absorbance = 10; final ferricyanide concentration = 60 mM. Limiting currents determined by chronoamperometry at E_app = +450 mV (vs. Ag/AgCl).

3.4. Dynamic working range

The working range of the assay was investigated using various dilutions of the GGA and OECD standard solutions, over 3 (Fig. S-2) and 6 h (Fig. 3) incubations and on three separate occasions using the OECD standard (i.e. using sludges glutamic acid proceeds at a slower rate (3−6 h). This is not uncommon as a number of microorganisms, such as E. coli, are known to preferentially and rapidly oxidize glucose before all other substrates, as glucose causes catabolic repression of other carbon and energy source use (Catterall et al., 2001; Morris et al., 2001; Pasco et al., 2004; Tortora et al., 1997). These findings are significant given the complexity of the OECD standard, which is composed of a range of substrates of varying lability and therefore better represents WWTP influents that the activated sludge community is typically exposed to. For these reasons, a number of other rapid BOD studies have used the OECD standard in preference to the GGA standard (Kumlanghan et al., 2008; Liu et al., 2000; Velling and Tenno, 2009; Yoshida et al., 2001, 2000). Although not shown in Fig. 2, since the endogenous control values have been subtracted, the assay is restricted to a minimum incubation time of 3 h, below which the endogenous contribution to the overall signal becomes excessively large, as high as 89 ± 5.7 and 91 ± 2.8% for the 15 min incubation with the GGA198 and OECD170 standards respectively. In comparison, for a 3 h incubation the endogenous proportion fell to 68 ± 5.0 and 70 ± 5.9% for the GGA198 and OECD170 standards respectively and further declined to 66 ± 5.0% for both standards after 6 h incubation. Further validation of the calibration standards and incubation time for the activated sludge FM-BOD assay will be investigated in the following two sections.

Fig. 3 – Determination of the 6 h activated sludge FM-BOD dynamic working range, using the GGA and OECD standards as the test substrates. The OECD working range was replicated on three separate occasions (closed symbols; solid lines). The GGA working range is displayed also (open symbols; dotted line). Endogenous control values have been subtracted. RAS was used as the biocatalyst; final sludge absorbance = 10; final ferricyanide concentration = 60 mM. Limiting currents determined by chronoamperometry at E_app = +450 mV (vs. Ag/AgCl).
collected on three different days). Close examination of the data reveals that each of the 6 h OECD standard curves remain linear to ~80 mg BOD$_5$ L$^{-1}$ and the 6 h GGA standard curve remains linear to ~100 mg BOD$_5$ L$^{-1}$. The linear working range for the 3 h incubation is approximately half that reported above for both standards. These reportable linear ranges compare favorably with the BOD$_5$ assay and rapid O$_2$ based BOD biosensors but poorly with previous FM-BOD studies using pure cultures of microorganisms (0–300 mg BOD$_5$ L$^{-1}$) (Morris et al., 2005).

It is apparent that the trend of substrate degradation remains similar, albeit at different magnitudes depending upon the specific sludge activity for the given day (Fig. 3). As expected, these patterns conform well with the Michaelis-Menten model (Fig. 3; Eq. (3)) (Lehninger, 1970). At low substrate concentrations, the reaction rate is first-order, beyond which point the reaction rate starts to decrease and is no longer linearly proportional to the substrate concentration. By basing the dynamic working range of the assay upon adherence to the Michaelis-Menten model, the working range can be extended beyond linearity and compares well with the previously published FM-BOD literature. This is a more relevant approach for deriving FM-BOD equivalent values when the linear range is small rather than reliance upon a single point calibration and the assumption of linearity as previous FM-BOD studies have done, especially given the innate variability of activated sludge activity (Fig. 3) compared to pure cultures grown under standard conditions in the laboratory. For the OECD and GGA standards respectively, the range extends to approximately between 10–170 and 10–300 mg BOD$_5$ L$^{-1}$ for the 6 h incubation (Fig. 3) and 14–80 and 14–200 mg BOD$_5$ L$^{-1}$ for the 3 h incubation (Fig. S-2). The lower limit of the assay working range is derived from the FM-BOD limit of detection. Derivation of real sample FM-BOD equivalent values using Eq. (3) requires dilution to within these ranges and at least a four-point calibration curve (including zero).

### 3.5. FM-BOD and BOD$_5$ comparison

The relationship between FM-BOD and standard BOD$_5$ measurements was investigated for 35 industrial wastewater effluents of high diversity (with BOD$_5$/COD ratios ranging from 0.16–0.76; mean 0.52 ± 0.13) and variable BOD$_5$ values (Table S-2). Calibration of FM-BOD equivalent values employing the OECD standard at longer incubation times (i.e. 6 h), provided the most robust correlation (Fig. 4).

The strength of this relationship is noteworthy, given the high correlation coefficient and the slope of the principal axis of the correlation ellipse (1.07) approaches unity. Although on average the FM-BOD measurements are slightly underestimated, the 95% confidence intervals of the correlation ellipse range between 0.96–1.19. Based upon this, the mean variability of the FM-BOD/BOD$_5$ relationship is ±10.7%, which represents an improvement upon the recognized variability associated with the BOD$_5$ assay alone of ±15% (APHA, 1999). It is therefore likely that a greater proportion of the variability evident in Fig. 4 arises from the BOD$_5$ measurements, especially considering the FM-BOD variability (i.e. R$^2$ values) observed in Fig. 3.

The mode of deriving FM-BOD equivalent values (i.e. calibration based upon the Michaelis-Menten model) was validated by comparison with BOD$_5$ measurements. Given the logistical restraints imposed primarily by the BOD$_5$ method, the 35 industrial effluent samples were incubated and analyzed in four separate batches (on separate days), thus using four different individually calibrated, activated sludge seeds (Fig. 4). Yet, in spite of intrinsic temporal variability with sludge activity (Fig. 3) a strong relationship between the two bioassays was still observed. Closer scrutiny of the four separate data sets in Fig. 4 reveals that no one data set deviates markedly from unity or the other three sets. This finding is significant given the range of BOD$_5$ values measured, the diverse nature of the samples analyzed, the different activated sludges employed and the inherent variability of the BOD$_5$ assay.

Incubation time and the relative degradability of the FM-BOD calibrating standard were integral in predicting BOD$_5$ values. Irrespective of the standard used for FM-BOD calibration, the relationship with BOD$_5$ data was improved using a 6 h incubation (Fig. 4, Fig. S-3) compared with a 3 h incubation (Fig. S-4). Assays employing the OECD standard were superior predictors of BOD$_5$ values for the majority of industrial effluent samples analyzed in this study. The 3 h GGA incubation was the poorest predictor of BOD$_5$ values. This was in keeping with the readily biodegradable nature of the GGA standard, particularly over the first 3 h of incubation, relative to the OECD synthetic wastewater standard (Fig. 2). Calibration with GGA typically resulted in overestimating the BOD by a factor of almost 2, particularly for readily biodegradable, high range samples (Fig. S-4b). This effect is also evident with the 6 h GGA incubation, although to a lesser extent, as it is ameliorated by the increased incubation time (Fig. S-3). The suitability of
which standard to use in an industrial application will depend upon the nature of the majority of wastewaters that would normally be analyzed for $\text{BOD}_5$. For example, if the $\text{BOD}_5$ values of industrial effluents are known to be relatively low (i.e., $<1500 \text{ mg } \text{BOD}_5 \text{ L}^{-1}$) and unchanging, a 3 h incubation using the OECD solution as the calibrating standard would be sufficient (Fig. S-4a). If on the other hand, $\text{BOD}_5$ values are relatively unknown, variable and/or high range, a 6 h incubation would be far more suitable, calibrated with the OECD standard for relatively biodegradable samples or with the GGA standard for relatively recalcitrant samples. For best results, application of the activated sludge FM-BOD assay in WWTPs requires some knowledge of the nature of the industrial effluent samples to be analyzed and ideally some optimization of the method to customize it to the given WWTP sludge.

The $\text{BOD}_5$ assay has a number of strengths and weaknesses. Most notably it boasts a high degree of substrate oxidation (60.5%) and yet achieving this requires five days (APHA, 1999). The strength of the relationships outlined above indicate that the activated sludge FM-BOD assay is capable of oxidizing a wide range of organic substrates in complex matrices, much the same as the $\text{BOD}_5$ assay does over a five-day period, but over a considerably shorter time-frame. Comparing the extent of biooxidation of both methods confirms this. Mean FM-BOD substrate oxidation of all 35 industrial effluent samples compares very favorably to the amount of biooxidation observed in the $\text{BOD}_5$ assay, for the 3 h (80 ± 22%; ranging from 43 to 130%) and 6 h (96 ± 23%; ranging from 62 to 167%) incubations. These values were calculated by comparing the amount of electrons that were biochemically transferred to ferricyanide during the FM-BOD incubation (by measuring ferrocyanide production) with the amount of electrons biochemically transferred to $O_2$ in the $\text{BOD}_5$ assay (by measuring $O_2$ consumption) (Catterall et al., 2003; Morris et al., 2003). This high level of substrate oxidation is possible given that essentially the $\text{BOD}_5$ and FM-BOD assays use the same diverse microbial community. However, the FM-BOD incubation time is greatly reduced, since the terminal electron acceptor is no longer rate limiting and much higher microbial concentrations can be employed. Maximizing the % oxidation of a wide diversity of real samples is integral to confident prediction of $\text{BOD}_5$ values using any rapid $\text{BOD}_5$ surrogate assay. It is this fundamental aspect of rapid BOD estimation that inherently limits BOD biosensors. While BOD biosensors are very rapid, typically the % substrate oxidation is <1% (Morris et al., 2001), therefore considerably overestimating the readily biodegradable fraction of industrial wastewaters. Previous FM-BOD studies (Catterall et al., 2003; Morris et al., 2003) have for the most part, avoided this problem, by integrating a consortium (4–5 species) of pure microorganism cultures from diverse heterotrophic groups as the biocatalyst. Not unlike this study, these studies reported excellent relationships with $\text{BOD}_5$ measurements for a large number of industrial effluents, although the relative extent of biochemical oxidation in this study was superior. To our knowledge, no other rapid BOD assay has achieved relative degradation efficiencies comparable with this study.

The impetus for optimizing the FM-BOD assay with activated sludge as the biocatalyst was with a view toward its implementation in WWTPs, while still providing rapid results within a single working day. For practical purposes, the use of activated sludge as the biocatalyst requires a fraction of the time-consuming and expensive preparation of pure culture microorganisms, as it can be easily collected from the plant at any time. Perhaps more importantly, by using the same microbial assemblage in the FM-BOD assay as the WWTP activated sludge community, the FM-BOD assay provides a completely relevant and representative measurement of the BOD loading to the plant. Such inferences are impossible using pure culture biocatalysts or even judiciously selected consortia of pure cultures, as their metabolic capacities will be different to those of the activated sludge community in any given WWTP.

4. Conclusions

Although the FM-BOD approach is not new, this is the first time that activated sludge has been successfully demonstrated to be a suitable biocatalyst. This has several important implications. The BOD of complex wastewaters can be analyzed in a single working day, with a similar extent of organic biodegradation compared to the standard $\text{BOD}_5$ assay. The use of Michaelis-Menten equations for the calibration curve was also a novel development. Application of the FM-BOD assay in the wastewater industry would assist operators of treatment plants by providing timely data on problems with wastewater or effluent quality, allowing corrective actions to be made much sooner. By using activated sludge from each individual treatment plant, the FM-BOD assay provides a highly representative and practical result. Furthermore, as the composition of the activated sludge community changes over time, so will the FM-BOD response. The FM-BOD assay requires substantially less use of dilutions as the measurement working range covers three orders of magnitude. These features make the fast FM-BOD assay highly suitable for application in the extensive and diverse wastewater industry.

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Appendix A.

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.watres.2010.07.042.

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