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Ubiquity of activated sludge ferricyanide-mediated BOD methods: A comparison of sludge seeds across wastewater treatment plants

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ABSTRACT

Many studies have described alternatives to the BOD_5 standard method, with substantial decreases in incubation time observed. However, most of these have not maintained the features that make the BOD_5 assay so relevant – a high level of substrate bio-oxidation and use of wastewater treatment plant (WWTP) sludge as the biocatalyst. Two recently described ferricyanide-mediated (FM)-BOD assays, one for trade wastes and one for WWTP influents and treated effluents, satisfy these criteria and were investigated further here for their suitability for use with diverse biocatalysts. Both FM-BOD assays responded proportionately to increasing substrate concentration with sludges from 11 different WWTPs and temporally (months to years) using sludges from a single WWTP, confirming the broad applicability of both assays. Sludges from four WWTPs were selected as biocatalysts for each FM-BOD assay to compare FM-BOD equivalent values with BOD_5 (three different sludge seeds) measurements for 12 real wastewater samples (six per assay). Strong and significant relationships were established for both FM-BOD assays. This study has demonstrated that sludge sourced from many WWTPs may be used as the biocatalyst in either FM-BOD assay, as it is in the BOD_5 assay. The industry potential of these findings is substantial given the widespread use of the BOD_5 assay, the dramatically decreased incubation period (3–6 h) and the superior analytical range of both assays compared to the standard BOD_5 assay.

1. Introduction

Many studies have described fast alternatives to the BOD_5 standard method. Several approaches have shown promise, with great improvements in incubation time and analytical range, but they have not maintained the features that make the BOD_5 assay so relevant – a high level of substrate bio-oxidation and use of wastewater treatment plant (WWTP) sludge as the biocatalyst (see [1] and references therein). Ferricyanide-mediated (FM)-BOD assays boast high levels of substrate oxidation over incubation periods as little as 1 h [2,3]. The relevance and representativeness of FM-BOD measurements have since been improved by incorporating multi-species consortia as the biocatalyst [4,5]. However, WWTP activated sludge had not been successfully employed as biocatalyst until recently [1,6]. Largely due to use of activated sludge biocatalysts, significant and strong relationships were achieved between BOD_5 and FM-BOD measurements for a range of real wastewater samples: $n=35$, slope=1.07, $R=0.95$ using return activated sludge (RAS) as the biocatalyst with industrial wastewaters [6] and $n=33$, slope=0.94, $R=0.96$ using primary influent sludge (PIS) as the biocatalyst [1] for a mixture of WWTP influent, treated effluent, and greywater samples. Additionally, FM-BOD equivalent concentrations were determined within a single working day using both assays [1,6]. In the RAS FM-BOD assay, the biocatalyst was highly concentrated to maximize the analytical range and to achieve maximal substrate oxidation for the analysis of trade waste samples, which vary enormously in terms of composition, biodegradable complexity and BOD concentration. The PIS FM-BOD assay instead employed a much lower microbial concentration, to minimize the endogenous proportion of the FM-BOD measurement, in order to lower the limit of detection (LOD) of the assay to around that of the standard BOD_5 assay (2 mg BOD_5 L^{-1}) [7]. This made the more recently developed PIS FM-BOD assay of Jordan et al. [1] amenable to the measurement of low-range WWTP effluents and mid-range WWTP influents, the industrial application that the standard BOD_5 assay was principally designed for.

Abbreviations: APHA, American Public Health Association; BOD, biochemical oxygen demand; BOD_5, 5-day biochemical oxygen demand assay; FM, ferricyanide mediated; FM-BOD, ferricyanide mediated biochemical oxygen demand; GGA, glucose/glutamic acid; HACCP, hazard analysis and critical control points; LOD, limit of detection; MLSS, mixed liquor suspended solids; OD, optical density @ 600 nm; OECD, Organisation for Economic Cooperation and Development; PIS, primary influent sludge; RAS, return activated sludge; SSVI, stirred settled volume index; WWTP, wastewater treatment plant

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The metabolic activity of various sludges present in WWTPs is known to be inherently variable [8]. This is variability occurs both between WWTPs, given the considerable differences in bacterial diversity that arise due to differences in plant design and influent composition [9,10], and within plants, given intrinsic fluctuations of influent composition, environmental variables and manipulation of operating parameters over time. Despite this diversity in microbial assemblages and activities, the biological treatment component of all WWTPs is optimized to achieve efficient oxidation and assimilation of biodegradable organic carbon. The measure by which the carbonaceous removal efficiency of a WWTP is determined throughout the world is largely by means of the standard BOD_5 assay [7]. The BOD_5 assay typically incorporates activated sludge from a WWTP as the biocatalyst and therefore has to function well with the great diversity of sludges encountered. Therefore, to be effective and viable alternatives to this standard assay, new BOD methods must also be robust enough to allow sludges from a wide range of WWTPs to be utilized as the biocatalyst.

The principle aim of this study was to assess the ubiquity of application of the RAS and PIS FM-BOD bioassays by incorporating biocatalysts prepared from sludges collected from a number of WWTPs within the Gold Coast and Brisbane regions of southeast Queensland, Australia. It is anticipated that FM-BOD measurements will vary quantitatively from plant to plant and over time, along with sludge composition, but that when normalized against a known organic standard, calculated WWTP specific FM-BOD equivalent values should vary proportionately with BOD_5 values for a range of real wastewater samples. This would effectively demonstrate that both FM-BOD assays could be calibrated using a variety of sludge types and similar results could be obtained for the same wastewater samples using sludges from different WWTPs. This is an important step towards these bioassays being used widely within the wastewater industry, as the American Public Health Association (APHA) [7] states that alternative BOD assays may be employed where a proportionate relationship with the standard BOD_5 assay has been demonstrated.

2. Experimental

All reagents used in this study were of analytical grade and all dilutions were made using deionized (Milli-Q Element, Millipore) water. All reagents, samples and sludge biocatalysts were prepared according to the relevant assay [1,6]. For both assays, optimized conditions were used in this study and are summarized in Table 1.

2.1. Calculation of FM-BOD equivalent values

The concentration of microbially generated ferrocyanide was determined using chronoamperometry, as has been described previously [4,6], and is a measure of total FM-respiration. Net FM-respiration is determined by subtracting the limiting current for the control endogenous metabolism incubation from the sample/standard gross limiting current. Net respiration was used to calculate all FM-BOD equivalent values (see below).

Table 1
Summary of experimental parameters adopted in the RAS and PIS FM-BOD assays.

Exp. parameter	RAS FM-BOD [6]	PIS FM-BOD [1]
Biocatalyst	RAS	PIS
Pre-incubation conditions	Starved 24 h	Grown 24 h
Sludge conc. (OD)	10	0.25
Incubation time (h)	6	4

OD = optical density.

2.1.1. RAS FM-BOD bioassay [6]

FM-BOD equivalent values were determined according to Jordan et al. [6] where calibration data conformed to the Michaelis–Menten model. FM-BOD equivalent values were derived via a 3-point linear calibration according to Catterall et al. [4]. The OECD standard [11] was used to normalize most high range wastewater samples (i.e. >700 mg BOD_5 L^{-1}) and the GGA standard [7] for all mid-range wastewater samples (100–200 mg BOD_5 L^{-1}).

2.1.2. PIS FM-BOD bioassay [1]

FM-BOD equivalent values were derived from a 3-point linear calibration according to Catterall et al. [4]. The OECD standard [11] was used to normalize all moderate to high range wastewater samples (i.e. 10–500 mg BOD_5 L^{-1}) and the GGA standard [7] for all low-range wastewater samples (<10 mg BOD_5 L^{-1}).

2.2. Determining proportionality of FM-respiration responses with multiple sludges

2.2.1. Biocatalyst specifications

WWTP sludge (RAS and PIS) was collected from 11 different WWTPs in the southeast Queensland region. These WWTPs differed considerably in their capacities, design specifications, sludge concentrations (mixed liquor suspended solids (MLSS)), mean sludge age and sludge settleability (stirred settled volume index (SSVI)), and are therefore representative of a typical cross-section of WWTPs (Table 2). Given the diversity between the WWTPs, microbial community composition and metabolic activity was also expected to vary considerably.

2.2.2. Experimental design

Net ferrocyanide production (i.e. less endogenous control values) was used to determine net FM-respiration for all RAS

Table 2
General characteristics of each WWTP from which activated sludge was used as biocatalyst in this study. RAS and PIS were collected from each WWTP and prepared separately for each FM-BOD assay [1,6]. Design capacity represents maximal hydraulic loading during average dry weather flow conditions.

Plant #	Process	Design capacity ($mL\,d^{-1}$)	Mean sludge age (d)	SSVI ($ml\,g^{-1}$)	[MLSS] ($g\,L^{-1}$)
1	Oxidation ditch	93.2	13.5	78	4.10
2	5-Stage Bardenpho	17[a]	38.3	65	2.86
3	Oxidation ditch	15[b]	26.1	93	7.52
4	Oxidation ditch/mUCT[c]	57.5	12.9	107	4.07
5	5-Stage Bardenpho	7.5	15.6	86	5.32
6	Westbank	1.4	15.0	130	5.05
7	Oxidation ditch	7.5	23.2	103	3.56
8	Oxidation ditch	7.8	32.9	131	3.85
9	Primary sed + MLE[d]	28	15.0	98	3.93
10	Oxidation ditch	66[b]	19.4	107	5.19
11	No data available				

[a] Underloaded – receiving ~30% of capacity.
[b] Overloaded beyond capacity.
[c] Modified University of Cape Town.
[d] Modified Ludzack–Ettinger.

and PIS seeds from Section 2.2.1. OECD [11] substrate concentrations were determined by the calibration ranges previously described [1,6]. Substrate concentrations and microbial OD represent the final concentrations in the assay mixture.

2.3. BOD_5 seed preparation and analysis

BOD_5 concentrations were derived according to the APHA 5210B standard method [7]. RAS was collected as required from three different WWTPs in south-east Queensland (WWTPs # 1, 5 and 10 (Table 2)). These seeds were selected for logistical reasons. Following a 1500-fold dilution, each seed was prepared separately according to standard procedures [7]. Sample BOD_5 concentrations were determined using each of the three different seeds and are reported as mean values.

2.4. Comparison of FM-BOD and BOD_5 results for selected sludges

Mean $\pm$ 1 standard deviation (SD) WWTP sludge FM-BOD equivalent values and mean BOD_5 measurements were compared for 12 wastewater samples (RAS FM-BOD assay, 6 trade waste samples; PIS FM-BOD assay, 2 influent, 2 grey water and 2 treated effluent samples). Mean FM-BOD measurements were derived using 8 replicate sludge biocatalysts, 4 for each assay (PIS and RAS from: WWTPs # 3, 5, 6 and 10; Table 2). These sludges were judiciously selected to provide a typical cross-section of WWTPs, each varying considerably in terms of capacity, treatment process, sludge respiration activity (Fig. 1) and influent composition. Wastewater samples were diluted to within the working range of each assay [1,6] and FM-BOD equivalent values were calibrated with either the GGA or OECD standard, see Section 3.2 for specific details. The two paired sets of results were compared using the slope of the principal axis of the correlation ellipse, as described previously [4,5]. All assumptions were met and therefore the data was not transformed.

3. Results and discussion

3.1. Ferricyanide-mediated respiration data with different biocatalysts

Net FM-respiration was evaluated for both assays using RAS [6] and PIS [1] collected from all 11 WWTPs listed in Table 2. In every case, FM-respiration responded proportionately to increased substrate concentration (Fig. 1). This was established by regression line equations and R^2 values (obtained by forcing the line through the origin to represent subtraction of the endogenous response), with all R^2 values in the range 0.94–0.99. This demonstrated that the assays could be successfully calibrated without modification for sludges from each WWTP, even though they were originally optimized with a different sludge [1,6]. The magnitude of the response varied considerably between the sludge biocatalysts from different WWTPs, particularly for the RAS assay (Fig. 1b). Such a high degree of variability would be expected given the large differences in the characteristics of the WWTPs from which the sludges were sampled (Table 2), the consequent innate variability in the microbial communities [9,10] and their respiration activities [8]. However, this is of no detriment to either FM-BOD assay as pure substrate standards (OECD and GGA) are used to calibrate the FM-BOD equivalent values for each sludge biocatalyst used in the assays and in so doing, the results are normalized for the activity of the specific activated sludge utilized.

These results support previous studies that have sought to quantify differences in activated sludge respiration activity [12]. A recent study [13] found that FM-respiration, normalized for the

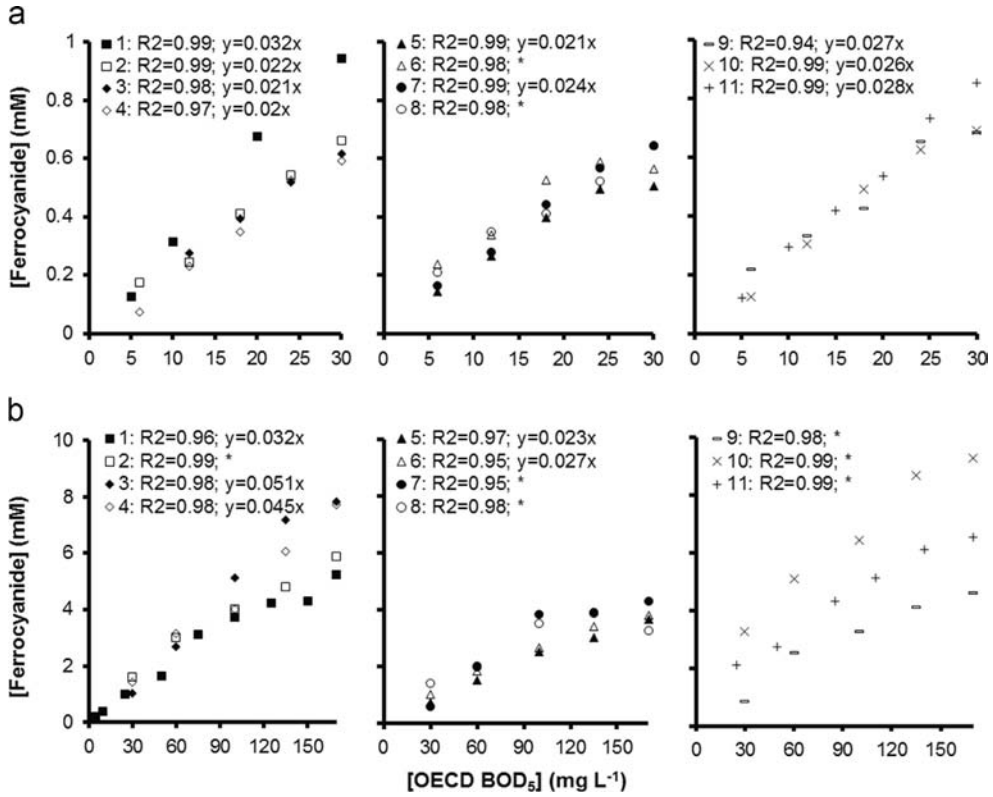

Fig. 1. Net ferrocyanide production as a function of OECD standard substrate concentration, for 11 different WWTP RAS and PIS sludge biocatalysts (from Table 2). (a) PIS FM-BOD and (b) RAS FM-BOD. Slopes and R^2 values are shown for sludges corresponding to the linear calibration model and R^2 values for those corresponding to the non-linear (hyperbolic) model (indicated by an asterix).

biocatalyst *Escherichia coli*, was not only dependent upon the microbial population density but additionally on the metabolic activity of the microorganisms at the time of incubation (i.e. their specific growth phase). No doubt this also contributes to the differences in activated sludge FM-respiration activities measured here and particularly when broadly comparing PIS (Fig. 1a) to RAS (Fig. 1b) activity. In the RAS assay, the overall rate of ferrocyanide production was comparable to that observed for the PIS assay. Although this is somewhat misleading, as the microbial concentration in the RAS FM-BOD assay is 40-fold higher than for the PIS FM-BOD assay (i.e. OD 10 vs. OD 0.25). Thus in reality, the PIS microbial community had a specific respiration rate ~40 times greater than that of the RAS microbial community. This is not surprising given the approximate ages of the FM-BOD biocatalysts and therefore the respective growth phases of the microbial communities. The mean RAS age of all 11 biocatalysts employed in this study (Table 2) was 21.2 ± 8.8 d. In comparison, the residence time within a typical sewer catchment, during average flow conditions, may only be ~6 h [14]. Therefore, evidently a large disparity exists between the RAS and PIS biocatalysts in relation to their respective growth phases. In WWTPs a long mean RAS age is not only encouraged but it is essential to ensure good clarifier settleability of the sludge [15]. Over such a long residence period, a large proportion of the microorganisms will be compromised, inactive or die (i.e. are in the stationary or death phase of growth). Whereas, microorganisms associated with the primary influent may typically be starved of oxygen but otherwise potentially very active. Upon exposure to surplus oxygen and substrate during the preparation of the PIS biocatalyst, the respiration activity of the sludge would rapidly increase due to increases in the specific activity of individual cells and growth of the cell population. This is typical of the lag and exponential phases of microbial growth.

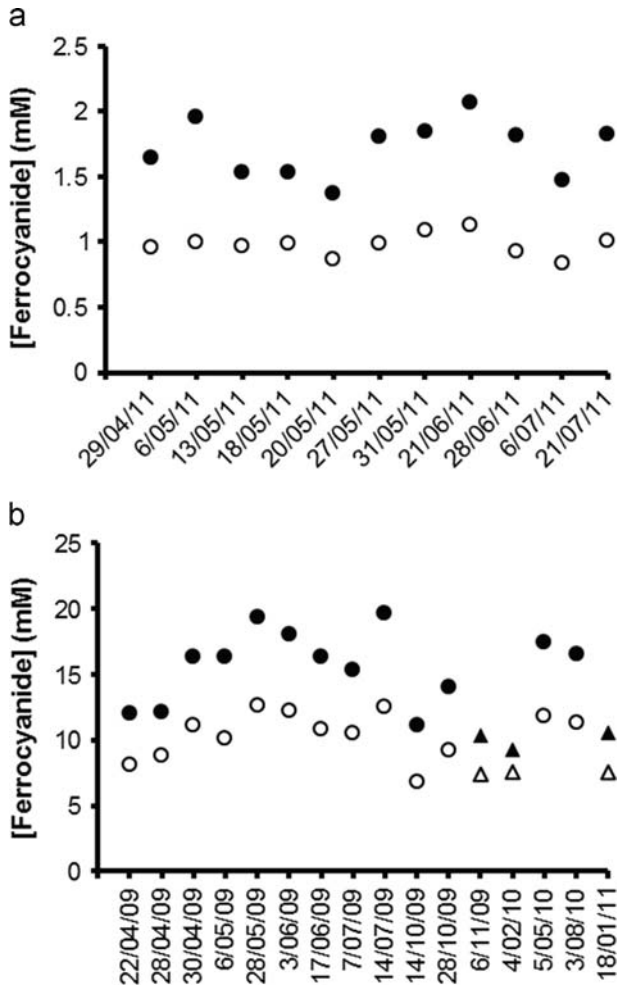

Fig. 2. Temporal variability of endogenous (open circles) and total (closed circles) ferrocyanide production of WWTP #1 (Table 2) sludges. (a) PIS FM-BOD, exogenous substrate = OECD[30] and (b) RAS FM-BOD, exogenous substrate = OECD[170]. Data corresponding to the Australian summer period are displayed as triangles. Note that the time series are over very different periods.

In most cases, FM-respiration increased linearly with increasing substrate concentration, to ≥ 24 mg BOD_5 L^{-1} (PIS FM-BOD assay Fig. 1a) and ≥ 135 mg BOD_5 L^{-1} (RAS FM-BOD assay Fig. 1b), although in some cases, especially for the RAS assay, a non-linear (hyperbolic) curve was more appropriate [6] (Fig. 1). The dynamic working ranges of both FM-BOD assays compare very favorably to those of the standard BOD_5 assay [7] and O_2 based biosensors [16]. The measurement range of these approaches is constrained by the solubility of oxygen (8.7 mg O_2 L^{-1} at 25 °C) and, consequently, analysis of wastewater samples requires a large number of serial dilutions, especially for trade waste samples, which typically vary greatly in organic concentration. An effective means to overcome this problem is by replacing oxygen with an artificial mediator, such as ferricyanide, that is considerably more soluble in water than oxygen and therefore not rate limiting. FM biosensors have been developed in the past [17–19] with linear working ranges comparable to or better than those reported for the RAS FM-BOD assay. However, in most cases only a single bacterial species biocatalyst was employed and given the extremely short incubation period, only a very low degree of substrate oxidation occurs during the analysis [2]. Therefore, these biosensors tend to overestimate the most readily biodegradable fraction of the sample, whereas more recalcitrant substrates are underestimated or are not measured at all [2].

Excellent linear working ranges have also been reported for single species FM-BOD assays [2], which are comparable to or better than those of the RAS FM-BOD assay. However, these assays also suffer from biased and non-representative BOD_5 equivalent values, as only a single microorganism biocomponent is employed. So as with single species biosensors, organic substrates present in the sample that are not utilized by the biocatalyst species are not measured. Moreover, in many cases the GGA standard has been used to determine the linear range of these assays [2,4], whereas in this study the more recalcitrant OECD standard was also employed. The OECD standard is formulated as a synthetic sewage analog and is generally regarded [19,20] to better reflect the composition and potential oxidation rate of real wastewaters, compared to the GGA standard. Non-linear (hyperbolic) relationships were observed in some cases, particularly for the RAS assay (Fig. 1). Non-linear calibration of FM-BOD equivalents were fit to the Michaelis–Menten equation [6]. Although in the majority of cases, a simple linear calibration will be sufficient, it is recommended that a 3-point calibration as a minimum should still be applied to confirm whether the use of a linear or non-linear model is more appropriate.

FM-respiration was not only observed to be inherently variable between WWTP sludge biocatalysts (Fig. 1), but also over time for a given WWTP (#1 Table 2). Measurements (endogenous and total respiration) over time with the PIS assay (Fig. 2a) indicate that the baseline metabolic activity was quite stable (mean ferrocyanide production = 0.98 mM ± 8.6% RSD, $n = 11$) while total respiration of OECD[30] had increased variability (mean ferrocyanide production = 1.7 mM ± 12.9% RSD, $n = 11$). This variability exceeds that which would be expected from analytical error, which has previously been determined from replicate ($n = 8$) OECD[5] analyses as ± 0.033 mM ferrocyanide (3.3% of the endogenous respiration and 1.9% of the total respiration). This observation suggests that there were changes in the microbial activity and community coming

into this WWTP over the period for which the measurements were made.

Measurements were also made with the RAS assay (Fig. 2b) over a longer period of time (mean endogenous ferrocyanide production = 10.9 mM ± 13.4% RSD; mean total ferrocyanide production of OECD[170] = 16.3 mM ± 15.3% RSD, $n = 16$) with a higher variability observed again. This data provides some insight into one possible reason for variability of RAS activity. Both total and endogenous FM respiration gave lower results in the Australian late spring and summer period (November–February), probably due to natural increases in a number of factors, namely temperature, organic loading and dilution due to increased tourist numbers and intrusion of stormwater during wet season summer storms. Similar variation of results was observed with GGA[198] standard solutions (data not shown).

Mean GGA[198] and OECD[170] BOD_5 data over the same period were observed to deviate from the standard values and the OECD standard in particular was quite variable (GGA[198] mean 175 mg BOD_5 L^{-1} ± 6.7%, $n = 11$; OECD[170] mean 199 mg BOD_5 L^{-1} ± 22%, $n = 11$). The mean GGA[198] value was on average 11.6% lower than the standard value, which is an acceptable degree of BOD_5 seed variability and yet BOD_5 sample concentrations are not adjusted to account for this [7] and would tend to be underestimated. A distinct advantage of using activated sludge FM-BOD assays compared to the BOD_5 assay is that the derived FM-BOD concentrations are directly normalized using a 3-point calibration with either the GGA or OECD standard. This calibration approach adjusts for any variability in the activity of the biocatalyst between WWTPs or through time.

3.2. Comparison of FM-BOD and BOD_5 measurements

Activated sludges from four WWTPs were judiciously selected to provide a typical cross-section of plants, for use as the FM-BOD biocatalysts in this section. RAS and PIS were collected from WWTPs 3, 5, 6 and 10 (Table 2). These plants varied considerably in terms of capacity, treatment process, sludge respiration activities (Fig. 1) and influent composition. For example, WWTP 5 has a higher proportion of industrial influent compared with WWTP 6 which receives 100% residential influent from its catchment. Specific influent composition data is lacking for most other WWTPs. At the same time, RAS sludge was collected from WWTPs 1, 5 and 10 (Table 2) as seeds for the standard BOD_5 assay.

RAS and PIS FM-BOD equivalent values and BOD_5 concentrations were compared using 12 real wastewater samples and the activated sludge biocatalysts outlined above (Fig. 3). Six trade waste samples were analyzed to evaluate the RAS FM-BOD assay, for which it was primarily designed. Similarly, 2 treated effluent, 2 primary influent and 2 gray water samples were analyzed to assess the efficacy of the PIS FM-BOD assay. Both FM-BOD assays reported highly significant relationships with the BOD_5 values determined for the same samples ($R = 0.99$ and $p < 0.001$ for both).

There was excellent agreement between the BOD_5 and the RAS FM-BOD assay results for trade waste samples (Fig. 3b), with a slope of 1.13 (Fig. 3b). The slope of the correlation for the PIS FM-BOD assay (Fig. 3a) was lower (0.70) indicating a consistent bias between the assay results, with the FM-BOD assay giving lower results. The most obvious explanation for this result is that the FM-BOD assay, with its lower overall degree of bio-oxidation compared to the BOD_5 assay, may be systematically underestimating the BOD of complex substrates. However, it was also evident that most of the variability associated with the relationship is due to variability between the BOD_5 measurements using the three different sludges seeds (±22.5%). This may at least in part be due to the differing calibration process, i.e. the FM assays are directly calibrated using a 3-point calibration for each series of

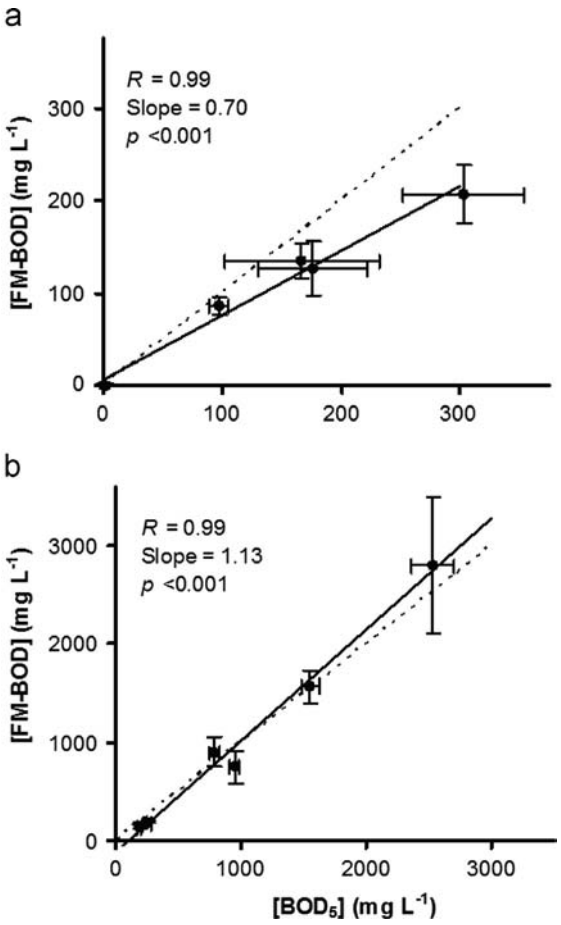

Fig. 3. Mean ± 1 S.D. relationships between the activated sludge FM-BOD equivalent values and BOD_5 measurements for 12 wastewater samples. (a) PIS FM-BOD and (b) RAS FM-BOD. The solid line represents the principal axis of the correlation ellipse and the dotted line represents the ideal slope of 1.

measurements, whereas the BOD_5 assay is indirectly calibrated against a single GGA standard (198 mg BOD_5 L^{-1} ± 15%), and BOD_5 values are not corrected to account for the associated error. As clearly visible in Fig. 1b, the RAS seeds (#1, 5, and 10) used in the BOD_5 assays varied considerably in their overall metabolic activity with sludge 10 having a much higher metabolic activity than sludges 1 or 5, and not correcting for this difference could be a considerable source of variation in the BOD_5 data. The lower degree of between assay variability for the four PIS utilized is a testament to the simplicity and robustness of the PIS FM-BOD bioassay, such that variability between FM-BOD measurements using different biocatalysts that we had no prior experience with was marginal. It will be important to undertake further comparisons of these two assays on these and other sample types to further investigate this relationship.

As has been detailed previously [1,21], accurate determination of real wastewater FM-BOD equivalent values, differing greatly in BOD concentration, may require calibration with more than one standard. The GGA standard was used as the calibrant for all relatively low-range wastewater samples (RAS FM-BOD assay: final $[BOD_5] = 185$ and 250 mg L^{-1}; PIS FM-BOD assay: final $[BOD_5] \sim 0.4$ and 1.2 mg L^{-1}) and the OECD standard was used to calibrate all relatively high-range samples, consistent with Jordan et al. [1]. The one exception being the FM-BOD RAS biocatalyst #6. This biocatalyst systematically underestimated the RAS FM-BOD equivalent values for each sample compared to the 3 other RAS seeds used in this assay, when calibrated with the OECD standard and therefore the GGA standard was also used to calibrate all

high-range values for this seed, as the GGA standard is known to be more readily biodegradable than the OECD standard [1,6,19–21]. This seed is the only one to come from a WWTP receiving no industrial (i.e. trade waste) influent at all, for which the RAS FM-BOD assay is specifically designed and is responsible for much of the FM-BOD variability associated with the high-range trade waste sample in Fig. 3b. Having not been exposed to such influents in the past, the #6 activated sludge microbial community may not oxidize some components of these wastewaters, which likely contain substances not present in domestic wastewaters. Therefore, prior to industry application of either FM-BOD assays for systematic analysis of wastewaters, it is recommended that analysts consider a pilot study comparing BOD_5 and FM-BOD equivalent values, calibrated with both the OECD and GGA standards, using a range of real wastewater samples ($n > 5$), in order to select the most appropriate calibrant for the sludge used in the FM assays.

3.3. Discussion of various FM-BOD bioassays

The standard BOD_5 and all of the FM-BOD bioassays described in the literature that have reported strong, significant relationships with the BOD_5 standard assay for a large number of real wastewater samples (n=30–35) [1,4,6] are compared with respect to key analytical characteristics and parameters (Table 3). The relative advantages and limitations of each assay type are discussed below.

3.3.1. Biocatalyst

The analytical signal of any bioassay or biosensor is inherently dependent upon the representiveness of the incorporated biocatalyst. The spectrum of substrate bio-oxidation provided by pure cultures of microorganisms or even judiciously selected consortia of pure cultures, cannot match that of WWTP sludges that contain a wide biodiversity of microorganisms, which naturally evolves and adapts to the composition of the influent wastewater [22]. This represents one of the foremost limitations of pure culture/consortia FM-BOD methods that typically employ only a single or a few species of microorganisms as biocatalyst, as any substrate present in the samples analyzed, which is not respired by the biocatalyst will not be measured in the analysis. Therefore, these methods will tend to underestimate the BOD of complex samples; to account for this they are calibrated with a readily biodegradable standard, such as GGA [4]. Although, activated sludge has been successfully immobilized within biosensors and biocoils [20,23–26] their practical application for legislative purposes may be limited. Largely due to significant changes in bacterial community composition over time that can arise during preparation and storage of biosensors/biocoils under conditions (selective pressures) that differ considerably from those in WWTPs [16]. Therefore, only the RAS and PIS FM-BOD bioassays, and the standard BOD_5 bioassay, can be considered to utilize truly representative biocatalysts, as these assays employ natural WWTP microbial communities. These assays entail simple preparation procedures that are unlikely to cause large changes in the composition of the microbial community. Moreover, the standard BOD_5, and as shown in this study the FM-BOD assays are highly versatile and can incorporate sludges from a wide range of WWTPs as the biocatalyst. This allows the BOD of an influent entering a WWTP to be assessed using a sludge from the same WWTP as biocatalyst, making the data obtained highly representative of the oxygen demand the influent will induce during treatment within the WWTP. This feature will ensure that a WWTP response to a sample can be predicted more accurately and the WWTP operation protected better as a consequence.

3.3.2. Biocatalyst preparation time

All preparation and analysis times are comparable for each FM-BOD assay (Table 3). However, any assay reliant upon the cultivation of pure culture biocatalysts is hindered by the time and effort required to prepare growth media, maintain cultures and grow the biocatalyst organism(s) in the laboratory. Employing a consortium of 4–5 pure cultures of microorganisms, as Catterall et al. [4] and Morris et al. [5] have done, extends this time further. Pure culture consortia have, in the past, been successfully freeze-dried or immobilized in polyvinyl acetate disks to negate this issue [21]. However, robust real sample relationships with the BOD_5 assay are lacking. Additionally, differential survival rates of the microorganisms during freeze drying and rehydration could result in shifts in the relative abundances of the microorganisms making up the consortium. Activated sludges on the other hand, can be easily sampled from any suitable WWTP at any time and rapidly prepared for the BOD_5 and FM-BOD assays.

3.3.3. Incubation time

The incubation period of all of the FM-BOD bioassays is rapid enough to allow the same day analysis of wastewater samples, providing WWTP operators with tools for timely identification of problems and the knowledge required for subsequent process optimization, if applicable. The five-day incubation time along with its limited analytical range, which necessitates multiple dilutions of unfamiliar samples, are the predominant shortcomings of the BOD_5

Table 3
Characteristics of the most promising FM-BOD assays [1,4,6] developed compared to the BOD_5 standard assay.

Assay parameters	Units	BOD_5 [7]	Consortium FM-BOD [4]	RAS FM-BOD [6]	PIS FM-BOD [1]
Biocatalyst		AS[a]	Consortia[b]	AS	AS
Biocatalyst prep. time[c]		✓	×	✓	✓
Incubation time	days; hours	5 d	3 h	6 h	4 h
Microbial concentration	OD	–	4	10	0.25
Working range	mg BOD_5 L^{-1}	1–7	50[d]	9.8–170[e]	2.1–40[f]
Limit of detection	mg BOD_5 L^{-1}	2.0	–	9.8	2.1
FM-BOD % biooxidation[g]	mean %	–	63 ± 12	96 ± 23	22 ± 8.7[h]

[a] Activated sludge.
[b] Pure culture microbial consortium of 4 species.
[c] See discussion for details.
[d] Linear curve; GGA standard.
[e] Hyperbolic curve; OECD standard.
[f] Linear curve; OECD standard.
[g] % Bio-oxidation relative to that measured by the BOD_5 assay. Value represents the mean of all real wastewater samples analyzed.
[h] % Bio-oxidation not determined for treated effluent samples as most were below the LOD.

assay. Efficient BOD removal is a major parameter for all WWTPs, with regulatory limits set for the BOD of treated effluents released to the environment or recycled for potable or other uses applying in most countries. However, given the long duration of the BOD_5 assay, it is not appropriate for monitoring or optimizing the efficacy of BOD removal in WWTPs and therefore other indirect indicators such as oxygen uptake rate are typically used as surrogate predictors [14]. Acceptance of FM-BOD techniques would provide the wastewater industry with a rapid and importantly direct measure of plant BOD removal efficiency and of the quality of treated effluents prior to their release or reuse.

3.3.4. Microbial concentration

This parameter is relatively trivial, that said, FM-BOD assays employing a high microbial concentration [6] require slightly longer and more meticulous biocatalyst preparation. This parameter is irrelevant for the BOD_5 assay.

3.3.5. Working range

The narrow working range of the BOD_5 assay is a second major constraint for its routine use. Any method with such a small analytical range will generate more labor, increased equipment demands and more guesswork for the analyst, to ensure that at least one serial dilution fits within the working range of the assay. Analysis of unknown trade wastes requires up to 5 serial dilutions of a sample [7]. The working ranges of the FM-BOD assays represent a 6-fold (PIS assay) and 25-fold (RAS assay) improvement upon the BOD_5 assay. Therefore, usually only 1–2 serial dilutions per sample are necessary for each FM-BOD assay. Capital and maintenance costs are comparable for the BOD_5 and activated sludge FM-BOD assays. BOD_5 labor costs are anticipated to be slightly higher, given the increased number of serial dilutions necessary.

3.3.6. Limit of detection

Treated wastewater effluents represent the majority of all BOD samples analyzed worldwide [7]. These samples are routinely analyzed to determine WWTP BOD removal efficiency and compliance with discharge licenses. Environmental regulatory bodies in Australia typically set lower limits for treated effluents of $\sim$10 mg BOD_5 L^{-1} for most WWTPs [27]. Some modern WWTPs and those around the world that have adopted the hazard analysis and critical control points (HACCP) early warning system, may typically apply much more stringent BOD_5 limits of $\sim$5 mg BOD_5 L^{-1}. Evidently, any BOD assay intended for routine analysis of these samples, must be accurate to at least this level and preferably, at least half that (i.e. $<2.5\ mg-BOD_5-L^{-1}$). This represents a principle strength of both the BOD_5 and PIS FM-BOD [1] assays (Table 3). LOD has not been optimized for any other FM-BOD assay.

3.3.7. FM-BOD % bio-oxidation

The BOD_5 assay boasts a very high level of actual to potential substrate oxidation for the GGA standard (60.5%) [7]. The values expressed in Table 3 are relative to the level of bio-oxidation observed for the BOD_5 assay, for the same sets of real wastewater samples. The high degree of substrate oxidation and low LOD of the BOD_5 assay make this assay suitable for analysis of both trade waste and treated effluent samples. For FM-BOD assays, the degree of sample bio-oxidation achieved and the assay LOD are largely a compromise. Achieving a similar level of substrate oxidation to the BOD_5 assay over a much reduced incubation period and yet maintaining a low microbial concentration (to reduce the endogenous signal proportion) [1] and therefore the LOD of the assay, is not feasible with the FM-BOD seeds investigated [1,4,6]. Thus, the consortium [4] and RAS [6] FM-BOD bioassays which employ high biocatalyst concentrations to maximize the assay working range are restricted to analysis of trade wastes, primary influents and higher range gray waters, as the seed has an inherently high endogenous respiration rate. Whereas, the PIS FM-BOD assay [1], has a smaller working range than the other FM, assays, although this is still 6-fold larger than that of the standard BOD_5 assay, as it employs a low biocatalyst concentration as this assay was primarily optimized for sensitivity (LOD) and it is therefore suitable for the analysis of treated effluents, gray waters, primary influents and even trade wastes after suitable dilution of the sample.

4. Conclusions

This study has sought to investigate the applicability of using activated sludge seeds sourced from a diversity of WWTPs as biocatalysts in the recently developed RAS and PIS FM-BOD assays. Despite manipulation of the activated sludge biocomponent in this study, solid FM-BOD to BOD_5 relationships are reported here for both assays using a number of real wastewater samples. This study provides strong evidence, that like the standard BOD_5 assay, both FM-BOD assays can also be utilized, employing activated sludge biocatalysts from any WWTP without any need for further modification. This degree of versatility has not been reported for any other alternative BOD assay incorporating WWTP activated sludge as the biocatalyst. Therefore, given the comparable or better characteristics of the FM-BOD assays compared to the BOD_5 assay, they represent exceptional surrogate BOD measures, which have considerable industry potential. Regulatory acceptance of any alternative BOD measure is understandably a major impediment to its widespread usage. However, the APHA [7] accepts that any suitable alternative BOD method may be used in place of the standard BOD_5 assay, where a proportional relationship has been established between the methods, as has been done here using a range of biocatalyst sources and water samples. This study therefore represents the first step toward FM-BOD regulatory acceptance.

Of the bioassays summarized in Table 3 (including the BOD_5 standard assay), the PIS FM-BOD assay [1] was found to be the most versatile and applicable method for routine industrial BOD measurement. On all but one criterion (% bio-oxidation) this assay either excelled above or was as good as the best of the other assays investigated. Due to assay LODs, only this assay and the BOD_5 assay can reliably be used for the statutory monitoring of treated effluents, which make up the majority of BOD samples worldwide. Moreover, the applicability of this FM-BOD assay has also been demonstrated with all other potential real sample types, with the exception of trade wastes.

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