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A Review of Practical Tools for Rapid Monitoring of Membrane Bioreactors.

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Abstract

The production of high quality effluent from membrane bioreactors (MBRs) arguably requires less supervision than conventional activated sludge (CAS) processes. Nevertheless, the use of membranes brings additional issues of activated sludge filterability, cake layer formation and membrane fouling. From a practical standpoint, process engineers and operators require simple tools which offer timely information about the biological health and filterability of the mixed liquor as well as risks of membrane fouling. To this end, a range of analytical tools and biological assays are critically reviewed from this perspective. This review recommends that Capillary Suction Time (CST) analysis along with Total Suspended and Volatile Solids (TSS/VSS) analysis is used daily. For broad characterisation, total carbon and nitrogen analysis offer significant advantages over the commonly used chemical and biological oxygen demand (COD/BOD) analyses. Of the technologies for determining the vitality of the microbial biomass the most robust and reproducible, are the second generation adenosine-5'-triphosphate (ATP) test kits. Extracellular polymer concentrations are best monitored by measurement of turbidity after centrifugation. Taken collectively these tools can be used routinely to ensure timely intervention and smoother operation of MBR systems

Key Words

MBR, Activated Sludge, process monitoring, CST, TOC/TNb, ATP,

Introduction

According to the Food and Agriculture Organisation of the United Nations (FAO) the volume of wastewater produced globally is unknown, largely due to a lack of data in many countries (FAO 2012). It is known however, that many hundreds of cubic kilometres of wastewater are produced. This immense volume of wastewater represents both a potential hazard, and a vital resource for humanity and the environment. In developed countries the bulk of this wastewater is treated in centralised plants where pathogen and nutrient content are reduced before it is either released into the environment or disinfected for reuse.

For over one hundred years the conventional activated sludge (CAS) process has been used to treat both domestic and industrial wastewaters (Lofrano and Brown 2010, Tilley 2011). This likely represents the largest use of industrial bioprocesses worldwide (Seviour and Nielsen 2010). Many permeations of the CAS process have been implemented to suit varying wastewater streams and local environmental conditions. CAS processes can suffer from biological phenomena such as bulking and reduced settling in clarifiers which result in poor quality effluent which contains suspended biomass (Jenkins, Richard *et al.* 2004).

The rise of membrane bioreactors (MBRs) over the last 15 years has been driven by the desire for smaller plant footprints, higher quality effluent, the necessity of water reuse and advances in membrane technology (Judd and Judd 2011). Although an aerobic MBR contains an activated sludge process, effluent suspended solids issues are mitigated by membrane separation. MBRs are an established technology now approaching maturity with 'fifth generation' plants currently being built (Kraemer, Menniti *et al.* 2012). MBRs can however fail to process the desired volume of wastewater if extreme caking, fouling or low biomass filterability occurs (Judd and Judd 2011). Additionally due to the necessity of constant membrane agitation, and chemical cleaning for removal of fouling, the capital and operational costs of ownership of MBRs is higher than for CAS processes (Le-Clech 2010, Kraemer, Menniti *et al.* 2012, Li, Yang *et al.* 2012).

Membrane fouling is typically categorised as either inorganic or organic, with the latter being less well understood. A large proportion of the literature regarding MBRs concerns mixed liquor properties and fouling propensity (Chang and Lee 1998, Rosenberger and Kraume

2002, Ng and Hermanowicz 2005, Pollice, Brookes *et al.* 2005, Rosenberger, Evenblij *et al.* 2005, Choi, Zhang *et al.* 2006, Le-Clech, Chen *et al.* 2006, Lebegue, Heran *et al.* 2008, Pan, Su *et al.* 2010, Tian, Chen *et al.* 2011, Ma, Wen *et al.* 2013). Research evidence strongly indicates that higher concentrations of extracellular polymers (ECPs) and / or soluble microbial polymers (SMP) are the keys to explaining low biomass filterability and a consequent high fouling tendency (Sheng, Yu *et al.* 2010). The literature is complex and marred by the variation in extraction methods and analytical techniques.

Given the uncertainties over operational issues affecting biomass filterability and the opex costs of MBR ownership, the question of how to best monitor the operation of an MBR and its biomass becomes more urgent. This review critically examines the range of analytical techniques (tools) now available for engineers and operators to monitor the biomass and treatment efficiency of an MBR. The techniques evaluated (Table 1 below) include both current analytical techniques as well as a range of newer techniques. In this review we have considered process chemistry methods for ion and sum parameter analysis and methods for the bulk assessment of solids. A particular focus is placed on the determination of extracellular polymers and viability and vitality by respiration indicators, dehydrogenase quantification and ATP measurement. Lastly, methods for microbial ecology are reviewed, with discussion limited to the number of techniques currently able to provide timely feedback.

Analysis	Main Application	Secondary Application	Notes
Ion Analysis			
Colorimetric	Compliance and process monitoring	n/a	Substantial historical use, well established parameters
Ion Chromatograph		Scale formation potential	Only viable for larger MBR plants
Sum Total Analysis			
COD	Compliance Monitoring Organics Reduction Evaluation & Mass Balance over processes	n/a	Substantial historical use, well established parameters
BOD		Influent toxicity monitoring	
TOC / TNb		Biomass C:N ratio monitoring	Emerging best available technology
Solids			
TSS/VSS	Inventory Management	Mass loading calculations	Substantial historical use, well established parameters
CST	MLSS Filterability & Dewaterability	MLSS Stress Response Detection	
Extra-Cellular Polymers			
Via Extraction	Fouling potential indicator	Tool for undersanding what operational conditions cause biological stress	Historical data is required to provide context for results
Via TOC			
Via Turbidity			
Biomass Viability and Vitality			
OUR	Biomass Health Assessment	Influent toxicity monitoring	Historical data is required to provide context for results
DHA - Tetrazolium		n/a	
DHA- Resazurin		n/a	
DHA - NADH		BNR DO control	
ATP (2nd Gen)		Biomass Stress Index (BSI) Active VSS ratio (AVSS)	BSI and AVSS comparable over different dates and treatment plants.
Ecology			
Microscopy	Filamentous bacteria monitoring	ECP monitoring	Historical data is required to provide context for results

Table 1: Evaluated analytical tools, their main and secondary applications in the operation of an MBR.

The analytical tools identified in Table 1 have been evaluated from a practical standpoint, and scored according to equipment and reagent cost, ability for point of testing use, ease of use and repeatability and timeliness allowing for rapid (same day) management responses. See Table 2 below for the scoring criteria and Table 3 for the results themselves. Although this review is largely focused on MBR operation, many techniques are transferrable to the operation of CAS plants.

Having placed a focus on the use of analytical tools in everyday plant operation, the review does not include the time consuming methods typically employed by researchers. For a review of molecular techniques in use for wastewater treatment the reader is directed to (Sanz and Köchling 2007, Seviour and Nielsen 2010). For the use of flow cytometery (Díaz, Herrero *et al.* 2010, Davey 2011) and for advanced image analysis of biomass (Costa, Mesquita *et al.* 2013). Moreover, this review does not cover the use of conventional on-line membrane engineering parameters such as monitoring of flux, permeability and trans-membrane pressure. For information on these see either the MBR Book or WEF Manual of Practice No 36 (Judd and Judd 2011, Water Environment Federation 2012). This review also omits the basic measurements of pH, Conductivity and Temperature, as they are assumed to be ubiquitous in all water labs.

NOTES ON THE CRITERIA FOR EVALUATION

Tools are evaluated under six categories, detailed in Table 2. Each category is rated 1 to 5 with 5 being the best.

Score Awarded	Reagents ($AUD per test)	Equipment ($AUD per test)	Location	Training	Confidence and Utility	Time
1	>101	>100,001	Large research facility (at a university or commerical provider of this service)	Requires full time personell dedicated to this equipment	Low repeatability (low confidence data) or results difficult to use without extensive research and prior results	>7 days
2	51-100	50,001-100,000	Large equipment, or requires a dedicated room (eg dark or clean rooms)	Requires scientifically trained personnel to oversee operation	Less repeatability (variation ~20%) or less usable results, where meaning of the test results is ambiguous	2-7 days
3	11-20	10,001-50,000	Well equipped on site laboratory (e.g. fume cupboard, incubators)	Requires extensive training. Best run by regular users	Moderate repeatability (variation ~15%), or where data is only suitable to guide operational decisions in context of past results	up to 1 day
4	6-10	5001-10,000	Larger portable equipment or basic on site laboratory	Can be performed by operators with moderate training	Reasonable repeatability (variation ~10%), gives clear process information	<1 hour
5	0-5	<5000	On site on portable or requires ubiquitous instrumentation	Can be performed by operators with little training	Highly repeatable (variation ~5%), suitable for immediate action/use	<10 mins

Table 2: Scoring matrix of key criteria detailed in the text

<u>Cost of Reagents & Capital Cost of Equipment</u>

The first two categories concern cost of the analysis. Firstly, the cost of consumable reagents and secondly the capital investment required. The lower these costs, the more likely the tests are to be incorporated into routine analysis. These costs may vary significantly depending on manufacturer, specification, volume purchased, purchasing power and location of purchase. The prices are in the experience of the reviewers, as very little in the way of price information is available publically. One exception is Hach Australia (http://au.hach.com/) where the recommended retail prices for most common wastewater equipment is available without signing in as an account holder. Please note, Hach do not sell all the analytical tools discussed in this review, nor are their products endorsed by the reviewers. Additionally, the retail prices given are likely to be higher than that actually paid by account holders. That being said, our ratings of cost have been given using these prices as a guide. Where cost of reagents vary significantly enough that they span several categories (for example colorimetric kit chemistry fall both below and above the $5 mark) half points will be used.

Location

In terms of producing data which can be used on the day of sampling, technologies which can be used onsite are clearly the most beneficial. The location score rates the requirements of the technology in terms of the sophistication of the laboratory required.

Time

The time required to perform each analysis or prepare it for automated processing is another determinant of suitability for routine use.

Training

Analysis which requires significant scientific expertise to perform is less desirable than that which are straightforward to use. To this end, procedures which are simple and little training is required are more highly rated.

Confidence and Utility

This category is designed to distinguish between tests with low repeatability and little clear operational meaning, and those which can be used with confidence and have well defined operational responses.

THE EVALUATION RESULTS

Analysis	Reagent Cost	Equipment Cost	Location Score	Time Score	Training Score	Confidence and Utility	Total Score
Ion Analysis							
Colorimetric	4.5	3.5	5	4	4	4	25
Ion Chromatograph	5	2	4	4	3	5	23
Sum Total Analysis							
COD	5	4	5	3	4	4	25
BOD	5	3.5	3	2	3	2	18.5
TOC / TNb	5	2	4	5	3	5	25
Solids							
TSS (MLSS) /VSS	5	4	5	4	4	5	27
CST	5	5	5	5	5	4	29
Extra-Cellular Polymers							
Via Extraction	4	3.5	3	3	3	3	19.5
TOC based bulk Characterisation	5	2	4	5	3	3	22
Turbidity based bulk Characterisation	5	5	5	5	5	3	28
Biomass Viability and Vitality							
OUR	5	4	4	5	3	3	24
DHA - Tetrazolium	3	3	2	3	2	3	16
DHA- Resazurin	5	4	4	4	4	3	24
DHA - NADH	3	3	2	3	2	3	16
ATP (2^{nd} Gen)	3	5	5	5	4	5	27
Ecology							
Microscopy	5	4	4	5	3	3	24

Table 3: Results of the Evaluation of MBR monitoring techniques against key criteria

INFLUENT / FILTRATE

CHEMISTRY

An established set of analytical chemical tests are routinely used to monitor both plant influent and filtrate to ensure that the required chemical transformations and nutrient removals are taking place. Without examining the internal process, data obtained from this 'black box' approach to process chemistry is able to give an operator confidence that there

are no major process upsets and that the effluent is likely to meet regulatory requirements for discharge. Typically, the analysis of plant influent and effluent include determination of various ions (e.g. NH_4^+, PO_4^{2-}) and sum total measurements (e.g. COD and TOC)

ION ANALYSES

Determination of nitrogen and phosphate ion concentrations is frequently carried out via colorimetric analysis using commercially available test kits. Whilst these kits are a quick and simple option, large or complex processes can require multiple parameters to be tested on many samples. In this case, the economic cost of test kit use can multiply rapidly given the cost and time involved in ordering, shipping, storing, actual use and finally disposal (Table 3). Different chemistries are available from a variety of suppliers at a range of costs typically between $2-$6AUD per test. The environmental cost of test kit use can also be quite high where vials are packaged in foam boxes or where the reagents themselves include hazardous chemicals.

A recommended alternative for high throughput, is the ion analysis using ion chromatography (IC). IC offers lower limits of detection (APHA, AWWA *et al.* 2005) and interference free analysis for highly coloured or sulfide containing waters, for which colorimetric determination is often unsuitable. IC can also quantify the full range of major cations (Li^+, Na^+, NH_4^+, K^+, Ca^{2+} and Mg^{2+}) and anions (Fl^-,Cl^-,Br^-,NO_2^-,NO_3^-,PO_4^{2-}, SO_4^{2-}) and can also be used to detect transition metals such as iron and manganese (Cardellicchio, Ragone *et al.* 1997). Although the capital expense of ion chromatography is significant, the advantages of having comprehensive on-site ion analysis are valuable for membrane processes which have a risk of (inorganic) scale formation. The ability to quickly check the Langelier Saturation Index (LSI) or Calcium Carbonate Precipitation Potential (CCPP), and adjust or initiate acid dosing could save many tens of thousands of dollars in cleaning chemicals, extend membrane life and reduce lost process time (Jefferies and Comstock 2001). Preparation of samples for IC is similar to that of test kit chemistry, with sample filtering and dilution of concentrated wastewaters. Through the use of an auto-sampler, IC determinations can proceed without the need for the continual attention of an analyst,

reducing staffing costs. The actual instrument time taken for the determination of each sample depends on the length of the columns used, but typically this would be between 15 and 30 minutes. Although ion analysis is important, the need to characterise plant influents and reductions across a processes, necessitates the use of 'sum total' analysis.

SUM TOTAL ANALYSES

Sum total analyses are tests which seek to give an overall assessment of a sample. These typically include BOD_5, COD and TOC. They are often used for estimating the organic load or removal efficiency of a process (Frimmel and Abbt-Braun 2011). BOD_5 limits frequently feature in in regulatory requirements (Higgins, Warnken *et al.* 2004). Conversion factors to determine one parameter from another (for example BOD from a COD measurement) should be used with extreme care and are not valid across different wastewater types (Aziz and Tebbutt 1980).

BOD_5

Biological oxygen demand (BOD_5) is a test of how much oxygen is consumed in a five-day period by microbes inoculated into a sample. It is thus a relatively crude indicator of the degradable material in a sample and is dependent on the viability of the microbial population seeded. BOD_5 has a substantial historical use (Jouanneau, Recoules *et al.* 2014), and is valuable for quantifying the potential biodegradability of a sample. For daily plant operation BOD_5 is impractical due to the five day incubation (and thus scores 2 for time) (Henze and Comeau 2008). Interferences to BOD can include ferrous iron, sulfides or reduced nitrogen compounds (Frimmel and Abbt-Braun 2011). In the latter case, the 'carbonaceous BOD' can be established by suppression of the nitrogenous bacteria with nitrification inhibitors. Unsurprisingly for a microbially mediated analysis, the variation in BOD_5 values for cross laboratory studies is up to 20% (Jouanneau, Recoules *et al.* 2014) and therefore scores 2 for confidence. For BOD_5 analysis of wastewaters with unusually complex or toxic properties, the use of standard microbial inoculum is likely to lead to under reporting due to the absence of metabolic capabilities required to degrade that sample (Jordan, Welsh *et al.* 2014). Despite the drawbacks of BOD it is useful to compare the COD:BOD_5 ratios of plant influent over time.

This can indicate a change in the treatability, or toxicity of the influent. Reagent costs are minimal (score 5), equipment requirements include incubators and dissolved oxygen or pressure measurement devices (score 3.5 for cost and 3 for location). The total score accorded to conventional BOD is 18.5. The search for a replacement technology which directly measures BOD_5 in a short time-frame or which can be used as an on-line sensor continues. Numerous faster methods, most popularly microbial fuel cells (MFC) (Abrevaya, Sacco *et al.* 2015) have been developed, and some commercialised (Namour and Jaffrezic-Renault 2010). As yet none of these has seen widespread uptake by industry possibly due to insufficiently rugged designs or unrealistic maintenance requirements.

COD

Chemical Oxygen Demand (COD) is a measurement of the concentration of organic compounds able to be oxidised using heat, dichromate and sulfuric acid. This test gives results in under three hours (therefore a time score of 3). Reagents are generally cheap amounting to $1-$2AUD a test (Reagent cost rate of 5) but disposal costs may also be in the same order of magnitude.

COD as a measurement of organic load can be artificially high in the presence of reactive inorganic species such as Fe^{2+} (Frimmel and Abbt-Braun 2011) or high levels of halides. Despite this, COD is the most commonly used method of assessment of oxidation demand (da Silva, da Silva *et al.* 2011). Whilst interference due to oxidation of inorganic compounds may not be a significant issue for domestic wastewaters, industrial wastewater may contain high concentrations of inorganic compounds in a reduced state (e.g. H_2S). In an aerobic MBR process H_2S will be oxidised to form SO_4^{2-} ions. Therefore, an organic mass balance based on COD will require influent and effluent measurement of the various sulfur species. For these reasons confidence and utility are rated a 4. Despite these shortcomings, COD is the most commonly used method of assessment of oxidation demand (da Silva, da Silva *et al.* 2011). The equipment required is a heating block and a spectrophotometer (score of 4) and is able to be done is even basic laboratory spaces (location score 5). The training required relatively

little however OHS&E aspects have to be stressed (training score 4). In our evaluation summary (Table 3) COD scores 25/30.

TOC

Given the shortcomings of BOD_5 and COD measurement there is growing movement to directly analyse Total Organic Carbon (TOC) compounds in wastewater (Aziz and Tebbutt 1980, Thomas, El Khorassani *et al.* 1999, Bisutti, Hilke *et al.* 2004, Gray 2010). The method utilised for the widest range of TOC analysis is High Temperature / Infrared (SM 5310 B), however for clean waters (ie MBR filtrate) the UV/persulfate method (SM 5310 C) has lower limits of detection (APHA, AWWA *et al.* 2005). TOC analysers which include simultaneous measurement of total 'bound' nitrogen (TNb) are available, making this a very attractive primary instrument for larger treatment plant labs. It should be noted that not all TOC analysers are sufficiently robust to handle samples with particulate material (Vanrolleghem and Lee 2003, Visco, Campanella *et al.* 2005), therefore samples run on these instruments require 0.45µm filtration prior to TOC analysis. The results would therefore more accurately be called *total dissolved organic carbon* (dTOC) rather than *total organic carbon* (TOC). More robust instruments with the ability to perform both TOC and dTOC will produce (via difference) the particulate TOC (pTOC), and therefore a much more complete picture of carbon movement and degradation through a treatment system. Additionally, the ability of a TOC analyser to handle particulate material means that it can be used to quantify the TOC (and TN if fitted) of both process waters and the biomass solids itself. This latter ability is useful to identify variations in normal carbon to nutrient ratios which may result in greater microbial polymer production and membrane fouling rates (Wang, Han *et al.* 2013). Whilst the confidence and utility are high (score 5), the capital cost of TOC analysis is the most significant detractor (score 2) however the cost of consumables is a minimal amount of high purity oxygen (score 5). Laboratory requirements are a benchtop space, access to bottle oxygen and reasonable grade pure water (Location Score 4). The training required is rated a 3 but is dependent on the product and its software. Sample preparation is quick (filtration for dissolved samples, dilution for sludges) and the use of an autosampler means many analyses

can be performed over a day without need for operator intervention (time 5). TOC is awarded a 24 /30.

Analysis of Mixed Liquor Suspended Solids (MLSS) Supernatant

In an MBR system analysis of the supernatant (in which solids are suspended) can provide important information either about nutrient removal or fouling propensity. For example, the ammonia, nitrate and orthophosphate levels in the supernatant taken from various zones can help diagnose the cause of nitrification / denitrification or phosphate removal issues. Typically the supernatant is separated from the biomass via centrifugation and then filtered (0.45um) prior to analysis (Rosenberger, Evenblij *et al.* 2005).

The supernatant may be significantly different to the filtrate of the MBR process in terms of organics and colloidal materials. This is due to the retention of all materials which are insufficiently soluble to pass through the MBR membrane. Depending on the pore size of the MBR membrane and the filter used to prepare the supernatant, a range of soluble and colloidal polymeric substances could be present.

The sampling and analysis of activated sludge supernatant requires more care than simply measuring the filtrate. Foam present on the top of MBR reactors can contaminate samples and care needs to be taken not to include foam in the MLSS sample. Secondly, centrifugation and filtering should occur as quickly as possible after sampling (Jenkins, Richard *et al.* 2004), or the results will be affected by continuing microbial removal of substrates. Phosphate accumulating organisms (PAO's) for example can release phosphate under low dissolved oxygen conditions. If supernatant analysis is performed regularly an operational "normal" profile can be established. Analysis showing deviations from this norm can assist in locating problems (such as loss of effective aeration or mixing).

Analysis of Extracellular & Soluble Microbial Polymers

The analysis of MLSS can also involve determination/characterisation of extra-cellular polymers (ECPs), a generic term for bio-polymeric substances. Soluble microbial products (SMPs) are the soluble fraction of ECPs. The measurement of ECPs/SMPs is discussed frequently in MBR literature particularly when the mechanisms of membrane fouling are under investigation. Many authors link ECPs/SMPs to low filterability conditions in MBRs (Drews, Vocks *et al.* 2008, Lyko, Wintgens *et al.* 2008, Pan, Su *et al.* 2010). The terms ECP and SMP apply to a wide range of molecules such as polysaccharides, proteins, nucleic acids, humic acids, lipids and other compounds which have been found at or outside the cell surface and in the intercellular space of microbial aggregates. This inclusive categorisation applies regardless of where these compounds originated. Disagreement exists over whether SMPs or ECPs play the more important role in filterability decrease and bio-fouling. Additionally, there are various opinions on the relative importance of protein or carbohydrate fractions. The often contrary nature of research findings is noted by other reviewers (Rosenberger, Evenblij *et al.* 2005, Drews 2010, Wang, Mei *et al.* 2013). It is also likely that some of the contrasting research findings are due to the variety in MBR designs, scale, design of experiments, membrane types and manufacturers, wastewater characteristics and the microbial populations present and active.

The exact definition and hence properties of ECPs and SMPs is heavily dependent on the methods used to obtain and characterize these biopolymers (Domínguez, Rodríguez *et al.* 2010a, Domínguez, Rodríguez *et al.* 2010b). ECP study is widespread however there are no standard methods for extraction making cross-comparison of study results difficult (Rosenberger, Evenblij *et al.* 2005). A comprehensive list of the various extraction methods can be found in (Sheng, Yu *et al.* 2010). Factors that negatively impact ECP studies arise initially in the extraction methods in which there is nearly always some degree of cell lysis and hence uncertainty over the actual amount of genuine ECPs in the original mixed liquor.

The origin and purpose of ECPs has also been the subject of much speculation. Firstly, ECPs can act as adhesives, assisting in floc and biofilm formation. In conventional activated sludge systems ECPs are considered vital for flocculation and their absence correlates with poor

settleability (Bala Subramanian, Yan *et al.* 2010). ECPs also serve as a protective barrier; providing resistance to toxins, temperature shocks and osmotic pressure changes and prevent desiccation. Thirdly ECPs assist in nutrient acquisition by sorption of organic compounds and metal ions, as well as assisting to retain enzymes involved in the digestion of exogenous macromolecules or those used for quorum communications (Raszka, Chorvatova *et al.* 2006). Lastly it has been noted by numerous authors that the production of ECPs increases when nutrient limitations occur, with most finding that the carbohydrate portion is particularly elevated (Janga, Ren *et al.* 2007, Sheng, Yu *et al.* 2010). There is also a consensus that lower temperatures (particularly shocks) tend to result in less biological degradation and a higher potential for colloidal material to accumulate and affect MBR filterability (van den Brink, Satpradit *et al.* 2011, Ma, Wen *et al.* 2013).

Once the extraction of ECPs or SMPs has been completed, it is typical for the protein and humic substances present to be determined according to the method described by Frølund *et al* (Frølund, Griebe *et al.* 1995), itself a modification of the classic protein only method of Lowry (Lowry, Rosebrough *et al.* 1951). The carbohydrate content of ECPs is often measured using another older traditional colorimetric method (Dubois, Gilles *et al.* 1956). In 2012 Silva and colleagues used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Matrix Assisted Laser desorption/ionization time-of-flight mass spectroscopy (MALDI -ToF/MS) to identify proteins isolated from bulk MLSS ECPs (Silva, Carvalho *et al.* 2012). The results of this study suggested that very few of the ECP proteins (~1% of soluble and none of the bound) were secreted extracellularly, and the vast majority were cellular break-down products. The conclusion to be drawn from this is that ECP formation occurs largely as a result of the death of microbial biomass. The concept that microbial cell death is an important consideration for the filterability of MLSS is supported by the work of several other authors (Hwang, Lee *et al.* 2008, Azami, Sarrafzadeh *et al.* 2011). Another study using MALDI- ToF/MS investigated the proteins found in the cake layer of MBR membranes identifying many as ‘sticky’ outer membrane proteins (OMP) of *Gamma* and *Betaproteobacteria*. (Zhou, Meng *et al.* 2015). At this stage the results for proteome studies of environmental samples are limited by the

databases of sequenced organisms, which are heavily dominated by the proteins of human related bacteria (Miyoshi, Aizawa *et al.* 2012). Attempts to use MALDI -ToF/MS to study polysaccharides in MBR fouling has so far been of limited success due to matrix effects (Kimura, Tanaka *et al.* 2012).

The lack of consensus on the causes of ECP production in the scientific literature is unsurprising given the variation in wastewater influent and microbial populations. It is likely that each MBR will have its own triggers for ECP production and heightened membrane fouling. Operational staff should monitor their MBR to elucidate which conditions correlate with ECP production. Whilst the characterisation of ECPs may be highly relevant to applied MBR research, regular operational determination of protein or carbohydrate portions is unlikely to be viable due to the time required for extraction and analytical characterisation (time score 3, training score 3, laboratory requirements location score 3, equipment cost 3.5), for a total of 19.5. We assigned all ECP / SMP methods a 3 for confidence and utility as results need to be interpreted in light of past results. Extraction free methods are likely to provide more timely data and so some authors have used measurements of the TOC or turbidity after centrifugation as a crude indicator of ECP/SMP content (Lyko, Wintgens *et al.* 2008). Bulk determination via TOC (reagent cost 5, equipment cost 2, location 4, time 5, training 3) for a total score of 22. ECP/SMP estimation via a turbidity assay is even more highly rated (reagent cost 5, equipment cost 5, location 5, time 5, training 5) for a total score of 28). ECPs in MLSS may be quantified by the use of direct light microscopy following reverse staining with India Ink (Jenkins, Richard *et al.* 2004). The subjective nature of this analysis without prolific replication is a significant drawback.

BIOSOLIDS ANALYSIS

TOTAL SUSPENDED SOLIDS (TSS) & VOLATILE SUSPENDED SOLIDS (VSS)

In MBR engineering and operational terms, the total microbial 'inventory' is usually taken as being the mass of the volatile suspended solids (VSS). The VSS is a subset of total suspended solids (TSS also commonly called mixed liquor suspended solids (MLSS)), and is effectively the organic compounds which volatilise at 550°C (APHA, AWWA *et al.* 2005). VSS is commonly used when assessing the food to microorganism ratio and managing solids inventory (Metcalf and Eddy 2003). The cost of each test is small (score 5) with GFC filter papers being only a few dollars (recommend 90mm diameter for MBR TSS). Laboratory requirements are a drying oven (105°C), a furnace (550°C) and a desiccator (location score 4). Although relatively simple this test requires careful laboratory technique, (the filter papers must be moisture free, and samples need to be weighed in a timely manner, particularly for low solids samples (training 4).

Despite the limitations of TSS and VSS measurements (discussed below), these measurements are the most valuable practical daily data for MBR operation and control (total score 27/30 in Table 3). In an MBR no measurable solids will exit with the effluent therefore regulation of the TSS/VSS depends entirely on the 'wasting rate' of excess sludge. High TSS/VSS loading (12-15000mg/l) is a recognised cause of significant cake layer formation (Le-Clech, Chen *et al.* 2006) and a dominant influence in the filterability of the MLSS.

The assumption implicit in the use of VSS as a measure of 'organisms' is that all the volatile material in the MLSS is microbial biomass. This is a problematic assumption on a number of levels. On a purely chemical level, the VSS removed at between 105°C and 550°C also includes chemically bound water, and the weight of the non-volatiles are impacted by the oxidation of inorganics. Additionally, phosphorous remains in the ash (as a 'non-volatile') despite originating inside cellular material. Another complication can be the accumulation of (non-microbial) organic compounds that are resistant to breakdown. ECPs are also produced and secreted into the MLSS by the microbial populations but could not be said to be living biology.

It is plain that TSS/VSS measurement are also insufficient to allow much understanding of the biological status of microbes in the mixed liquor (Andreottola, Foladori *et al.* 2002). In terms

of the actual microbial biomass in the reactor, a significant portion is not categorically 'alive' in that it not metabolically active (effectively dormant, or dead but intact), or alternatively alive but metabolising at an undetectable level. One study that biomass volume accounted for only 10-15% of the mixed liquor VSS (Frølund, Palmgren *et al.* 1996). Similarly a later (2010) study found that approximately 11.1% (on a COD basis) of the activated sludge was living biomass with only 45% of this being metabolically active (Foladori, Bruni *et al.* 2010). Whilst conventional solids testing is the backbone of MBR operation, it offers very little information of the actual viability, vitality and composition of the microbial population.

CAPILLARY SUCTION TIME (CST)

Capillary Suction Time (CST) is a simple chromatography based method used to measure how quickly the MLSS supernatant takes to travel between two points through a filter paper by capillary action. CST was initially developed as a tool to predict the potential to de-water sludge (dewaterability) (Gale and Baskerville 1967), it has since been found to be informative with regard to filterability of mixed liquor in an MBR and scores highest (29/30) in our evaluation summary (Table 3).

CST is better suited to evaluation of thickened sludge and MBR biomass than CAS biomass due to the higher MLSS concentrations (lower solids concentrations produce readings of less than 10 seconds which lack resolution). In MBR MLSS, the CST has a good demonstrated correlation with Specific Resistance to Filtration (SRF) and is a quicker test to perform (Chen, Lin *et al.* 1996, Higgins and Novak 1997, Scholz 2005).

The CST is influenced by the colloidal loading of the supernatant. As the sludge contacts directly with the filter paper, a 'cake layer' forms and acts as a barrier for further capillary action along the paper. The presence of macromolecules and fine particles which can increase the cake layer formed at the interface and hence increase the CST (Sawalha 2010). The concentration of suspended solids has a significant influence on the CST, therefore Standard Methods (2710 G) recommends normalising the result against TSS. This produces a CST in seconds per gram of TSS.

Although cheap (score 5 for reagent and capital costs) and quick (time score 5), CST can suffer from repeatability issues often due to leakage of the MLSS between the funnel and the paper (score 4 confidence and utility). A 2010 doctoral thesis investigated causes of CST variability and found that the type and pore size of filter-papers used, the temperature, and shape and size of the funnel all contributed to variation in the measured values (Sawalha 2010). Swalha recommended the use of a sealant between the paper and funnel to reduce the variability. An alternative solution is to run samples in triplicate and exclude outlying results.

Microbial Viability and Activity Tests

In order to move beyond the use of VSS as a measure of 'organisms' additional testing focusing on the viability and activity of microbes is required. These analyses can be based on respiration, cell membrane integrity, enzyme levels or cellular energy storage detection.

Respiration Based Tests for Microbial Activity

The oxygen uptake rate (OUR) also known as the oxygen consumption or respiration rate, is a simple measurement of the oxygen consumed by the total biomass. OUR as a monitoring tool, scores well in our evaluation largely due to low reagent and equipment costs (score 5 and 4 respectively) but does require careful implementation as outlined below. The specific oxygen uptake rate (SOUR) is the OUR normalised against VSS concentration. Additionally the portion of oxygen consumed by nitrification can be established by performing the test with and without a nitrification inhibitor (Strotmann, Keinath *et al.* 1995).

In the OUR test a fresh MLSS sample is infused with air until the DO reaches 4 mg/l. Once the DO drops to 3.0 mg/l a timed period starts and the oxygen depletion is monitored until the DO drops below 1mg/l. The slope of the decline, in conjunction with the VSS previously measured is then used to calculate the specific OUR or SOUR. Whilst this appears to be a relatively simple

test to perform, it has numerous opportunities for the production of unreliable data (training score 3), especially if a comparable result from multiple occasions is desired (a confidence and utility score of 3). Standard Methods 2710 B notes that the result is 'quite sensitive' to temperature, therefore replicate determinations need to be made at +/- 0.5°C, (necessitating a water bath). The test is normally performed at a temperature of 20°C, where this is not possible a mathematical compensation is able to be used. This compensation is only valid between 10 and 30°C. Additionally, the concentration of the sludge should be adjusted so that the test takes between 5-10 minutes. If the biomass is concentrated, insufficient data points will be obtained before the oxygen is depleted. In order to dilute the MLSS a (pre-warmed) buffer solution isotonic to the process supernatant should be used. Dilution may then result in settling out of the MLSS during the testing which can give uneven oxygen depletion. SOUR measurement will reflect the amount (or lack) of food in the wastewater supernatant, therefore some testing regimes will involve the addition of plant influent or acetate (Vollertsen, Jahn *et al.* 2001). The result is $SOUR_{MAX}$ which is often a more helpful measurement than SOUR (Archibald and Young 2004).

Even with these method adjustments, other authors have noted that that the reliability of SOUR data depends on quality and maintenance of the dissolved oxygen electrodes used (Gernaey, Petersen *et al.* 2001). This assertion is supported by the experience of the reviewers. So whilst the study of biomass oxygen consumption can be a cost effective method for monitoring biomass activity, the test needs to be performed with a great deal of care. The total score for is 24/30.

Alternative respiration measurements (not based on oxygen consumption) can focus on the evolution of, carbon dioxide or nitrous oxide (Xiao, Araujo *et al.* 2015). Measurement of these species is more difficult and expensive than oxygen consumption but may be more appropriate for MLSS treating specific wastewaters. For anaerobic MBRs methane production is a good indicator of microbial activity.

DYE BASED METHODS FOR VIABILITY AND VITALITY

There are a wide range of assays for the testing of cellular metabolism using dyes which react with metabolic products. There is also a range of dyes which can be used to determine membrane integrity (and thus distinguish living and dead cells). Largely these dyes are detectable by excitation at a specific wavelength and detection of the emission at another. Whilst many research publications have used these techniques to good effect (Ziglio, Andreottola *et al.* 2002, Berney, Vital *et al.* 2008, Foladori, Bruni *et al.* 2010), they are generally not able to be performed on-site. Typically, they require expensive laboratory equipment such as a fluorescence / laser microscope or a flow cytometer and staff with extensive experience in these methodologies. These techniques are powerful however because individual cells are visualised and counted automatically giving statistically significant data. These high tech methods are very sensitive and therefore analysis of 'non-ideal' sample types such as MLSS can be challenging. Typical hindrances are the tendency of biomass to clump together, the presence of unknown materials, uneven staining of microbial populations and significant background signals.

Some work has been done using dye based assay (LIVE/DEAD® BacLight™ Life Technologies) for microbial viability and activity on less complex platforms. A plate reader based assay to quantify the portion of living bacteria in probiotic solutions was used by Alakomi *et. al.* in which individual cells are not visualised or counted but red and green fluorescent signals are obtained for the total solution (Alakomi, Mättö *et al.* 2005). They found that each of the different bacterial strains required separate calibration curves. The inference is that LIVE/DEAD® BacLight™ Bacterial Viability Kit may not be suitable for mixed populations of unknown species. This view is supported by a study which investigated the proportions of live and dead bacteria in soil samples which proved unsuccessful despite complex mathematical compensations and careful dilution procedures (Pascaud, Amellal *et al.* 2009).

Dehydrogenase Activity (DHA)

A dehydrogenase is an oxidoreductase enzyme that oxidizes a substrate by a reduction reaction that transfers one or more hydrides (H^-) to an electron acceptor. Nicotinamide Adenine Dinucleotide (NAD^+) is reduced to $NADH_2$ (referred to as NADH) in one step of the electron

transport chain which generates ATP in bacteria. Measuring DHA is a direct measurement of this rate of conversion.

has been found to correlate positively with OUR and substrate removal in activated sludge (Awong, Bitton *et al.* 1985). DHA activity can be measured via a colorimetric or spectrophotometric analysis of the change of colour of tetrazolium salts or resazurin, as well as by direct measurement of NAD^+/NADH.

Tetrazolium salts

Tetrazolium salts (MTT (3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide), INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) are a group of compounds commonly used to measure dehydrogenase activity. Each of the tetrazolium salts can be reduced by dehydrogenase to a highly coloured formazin compound which can be measured using a colorimeter (Life Technologies 2010). The use of MTT and INT is limited by the insolubility in water of the reduced product, therefore XTT is a better option for a quick assay. Nevertheless the literature cites the use of INT for the determination of dehydrogenase activity in domestic biomass using a solvent extraction of the product compound (Liwarska-Bizukojc and Ledakowicz 2003). It was found that standard deviations of this method were quite high (3-15%) (resulting in a confidence and utlity score of 3). Using XTT as an indicator of dehydrogenase activity Bensaid and Thierie found a good correlation with OUR (r^2 = 0.977) (Bensaid, Thierie *et al.* 2000). The drawbacks of the XTT assay include the requirement for facilities for solvent extraction and sterile preparation of an electron decoupling reagent (location score 2, training 2), significant reagent preparation time and rapid expiry of these reagents (time 3) and therefore the high cost of each set of these reagents (reagent cost score 3). Combined, these factors result in a low evaluation score (16/30) in Table 3 for biomass monitoring via Tetrazolium salts.

Resazurin Reduction test

Resazurin dye (7-hydroxy-3H-phenoxazin-3-one-10-oxide) can also be used as an indicator of cellular dehydrogenase enzyme activity. The resazurin assay is moderately simple (time score of 4), cheap (reagent cost of 5) and can be performed using the visible spectral range of a spectrophotometer (equipment cost 4). Resazurin is blue in its oxidised state, when reduced to resofurin it becomes pink. The method first used in the 1950's for the determination of sperm health, was adapted as an assay to assess toxicity of chemicals by Liu (Liu 1986). This method involves a solvent extraction step, making it a slow and insufficiently rugged for on-site use. In 2007, McNicholl and co-workers modified this method to eliminate the solvent extraction step and simplify it. They found that it was then ideal for regular use on site at wastewater treatment plants (location score 4) (McNicholl, McGrath *et al.* 2007). Most recently the resazurin assay has been used to evaluate the potential for toxicity to sewage treatment plants from preservatives found in personal care products (Carbajo, Perdigón-Melón *et al.* 2015). Resazurin is the basis for the CellTiter-Blue® Cell Viability test kit (Promega) and also for the ToxTrak™ Reagent Set (Hach). The use of resazurin as a basis for toxicity detection in activated sludge is also detailed in the OECD Method 209 (OECD 2010). The numerous commercial uses of resazurin assays would seem to support its use as a viability and toxicity indicator, however Strotmann and co-workers found that "it did not always positively correlate with the simultaneously estimated respiration activity" (Strotmann, Butz *et al.* 1993). This is also the experience of the review authors, therefore a confidence and utility score of 3 and a total of 24/30.

Direct Measurement of NAD/H

Nicotinamide Adenine Dinucleotide (NAD) is involved in many biological oxidation/reduction reactions. It is alternately oxidised (NAD^+) or reduced to $NADH_2$ (NADH) by the loss or gain of two electrons respectively. The latter state can be detected when exposed to light at 340nm, and the fluorescence measured at 460nm. In a laboratory study the fluorescence detected was found to be proportional to the NADH concentration (König, Berns *et al.* 1997). Studies using florescence probes outside the lab claim to provide a direct measurement of cellular metabolic activity (Armiger, Lee *et al.* 1994, Farabegoli, Hellinga *et al.* 2003). Several studies have

outlined NAD/Hs on-line use potential for controlling dissolved oxygen in biological nutrient removal (BNR) where alternating aerobic and anoxic zones are required (Armiger, Lee *et al.* 1994, Parikh, Trivedi *et al.* 2011). An additional study found that the probe was a rapid indicator of fermentation imbalance with (anaerobic) reactors subjected to five different perturbations (Peck and Chynoweth 1992). Others however were slightly more reserved about the potential noting that NADH monitoring 'proved not to be as straightforward as suggested in the literature'(Farabegoli, Hellinga *et al.* 2003). This view is supported by Wos and Pollard who commented that both the studies supporting NADH probe use failed to separate extracellular from intracellular NADH (Wos and Pollard 2006). Furthermore that Armiger and Lee *et al* did not address matrix issues such as turbidity which can cause light scattering thus having a negative impact on excitation of the NADH and detection of the emitted fluorescence (, fluorescence quenching) resulting in a significant under reporting of NADH (Wos and Pollard 2006). These concerns would be amplified when analysing MBR biomass due to the higher MLSS concentrations commonly used. Finally, there are currently no vendors of the on-line NADH process control equipment mentioned in the literature; Biobalance (Denmark), Fluoromeasure (BioChem) and Fluorosensor (Ingold)). There are a number of *ex-situ* assay measurements available, none of which are designed for wastewater matrices. Alternatively, a procedure developed for wastewater biomass by Wos and Pollard can be performed however this requires care in the making of reagents and standards (training score 2, time score 3, reagent cost 3) as well as a fluorimeter (equipment cost 3, location 2) (Wos and Pollard 2006). These factors explain the low score for NADH assays of 16/30.

ADENOSINE-5'-TRIPHOSPHATE (ATP)

Adenosine-5'-triphosphate (ATP) is the main energy molecule of living organisms (excluding viruses) and therefore the detection of ATP is indicative of cellular life. In the food industry ATP measurements have been widely used to evaluate the success of sterilisation techniques (Powitz 2007).

Numerous test kits requiring a luminometer are available commercially for biotechnology laboratory use; BacTiter-Glo™ (Promega), EnzyLight™ (BioAssay Systems) and the Calbiochem® ATP Assay Kit (Merck Millipore). These are all bioluminescent ATP Assay kits used to determine the amount of ATP in a sample and thus the vitality of the microbial life present.

There are a number reports in the literature of the use of ATP tests for analysis of biological activity in activated sludge, however most are dated pre 2000. Early work indicated that ATP was a sensitive measurement of biomass viability (Patterson, Brezonik *et al.* 1970, Weddle and Jenkins 1971, Jørgensen, Eriksen *et al.* 1992). Later work demonstrated that ATP analysis was an instructive parameter for warning of toxic influents, undesirable process changes (Arretxe, Heap *et al.* 1997, Dalzell and Christofi 2002) or determining the impact of pharmaceuticals on MBR biomass health (Maeng, Choi *et al.* 2013). Despite these successes the use of ATP tests in the daily operation of wastewater treatment has not been widespread due to the complexity of wastewater matrices and the high level of scientific training required.

The analysis of biomass health can be made by determining the ATP content per gram of VSS. Although some variations in the ATP content per cell have been reported (due to differences in growth phase, cell size and microbial species), it is recognised as being more consistent for cellular enumeration than other measurements such as protein, DNA, particulate nitrogen or the fluorescent stains DiBAC4(3) and carboxyfluorescein (Weddle and Jenkins 1971, Berney, Vital *et al.* 2008). Studies using flow cytometry have found an average ATP-per-cell value of 1.75×10^{-10}nmol/cell (Hammes, Goldschmidt *et al.* 2010).

Recently a range of test kits have been developed, including one specifically designed for activated sludge health assessment (LuminUltra QG21W). Unlike first generation technologies (mentioned above), this 2nd generation test measures both total and cellular ATP, so the extracellular ATP content can be calculated. The proportion of ATP inside cells compared with outside the cells (in the supernatant) can be used to measure the the biomass stress index (BSI). This dual determinaiton addresses a major limitation of most ATP test kits, as noted by Hammes *et al* (Hammes, Goldschmidt *et al.* 2010). Although not a cheap option (with a regent score of 3), the advantages of this test kit are; a large sample size can be taken, the equipment

is portable and easy to use and the procedure is quick (<5 minutes), giving scores for location of 5, equipment cost of 5, and a training score of 4 and time score of 5. Standards included with the kit enable quantification and comparison of results over time (confidence=5) giving a final score of 27/30 in Table 3. Keasler *et. al.* reported the use of a 2nd generation ATP test kit to assess the microbial content of oil-field systems (Keasler, Bennett *et al.* 2012). In their study ATP based determinations (used on the basis of 1 femtogram of ATP/cell) and quantitative PCR (see below) were in good agreement, whilst conventional serial dilution greatly underestimated the populations. A related test kit has also successfully been used for the early warning of activated sludge bulking/foaming (Brault, Whalen *et al.* 2011).

MICROBIAL ECOLOGY

MBR processes can be affected by the amount of biomass, the activity of the biomass and the microbial composition of the biomass. In an effort to understand the latter, a range of molecular microbial ecology methods have been developed. The most promising of these is Next Generation Sequencing (NGS), which is capable of providing insights into the composition of microbial communities and in some cases the metabolic consequences of this composition (Albertsen, Hansen *et al.* 2012, Sekiguchi, Ohashi *et al.* 2015, Beale, Karpe *et al.* 2016).

Whilst the cost of NGS has vastly reduced, and bioinformatics tools are becoming more user friendly, currently NGS cannot offer timely results for operators. However, given the rapid development of this field, in particular in 16s based molecular technology, there is hope that in the future this technology may provide water engineering relevant tools. Meanwhile basic microscopy is the best tool available.

MICROSCOPY BASED ECOLOGY

Direct examination of microbial population using a light microscope coupled with specific stains/dyes has long been a mainstay of wastewater treatment plant microbiology (Eikelboom 1975). An experienced operator can identify morpohotypes (bacteria with the same appearance) regularly present and conversely any changes to the normal micro flora of the

MLSS. Given the relatively distinct morphotypes broadly associated with bulking and foaming (Wanner and Grau 1989, Jenkins, Richard *et al.* 2004) the use of microscopy is understandably widespread (Seviour and Nielsen 2010).

Light microscopy does represent a quick (time score of 5) and cost effective means of monitoring biomass (reagent cost of 5), especially with the price of electronics and software reducing rapidly (equipment cost 4). The microscope should include phase contrast and a 100x oil immersion objective. To make the best of this technique the microscopist requires experience and familiarity with the specific WWTP biomass (training score of 3). The use of stains to assist in identification may not always be helpful. For example the presence of sulfide can result in Gram variable and Neisser variable results for some morphotypes such as *Thiothrix* and *Nostocoida* species (Jenkins, Richard *et al.* 2004).

With the advent of molecular methods for identification of microbes, the inexact nature of morphological typing became has become evident (Müller, Schade *et al.* 2007). For example some filamentous organisms are able to revert to a unicellular form at some stages of their lifecycle (Ramothokang, Naidoo *et al.* 2006). Other morphological groups such as 'Eikelboom Type 1863' have been shown to comprise of several unrelated taxa (Seviour, Blackall *et al.* 1997) (therefore a confidence and utility score 3). Although clearly basic light microscopy has its limitations, it is currently the only practical microbial ecology tool available (overall score 24/30)

CONCLUSIONS

Timely and dependable analyses are vital for smooth and cost efficient operation of an MBR, we have reviewed and evaluated many different technologies with a view to establishing a standard set of timely and reliable analytical tools for monitoring and regulating MBRs. The choice of monitoring technologies to employ for an MBR and its biomass must be considered carefully and Table 3 summarises the relative advantages and disadvantages of the different technologies assessed against factors such as cost, time, suitability for on-site location, required training and the usefulness of the result.

In summary, TSS/VSS measurements will always remain important parameters, but for MBR operation CST is also very valuable. For less equipped plants colorimetric analysis (including COD) is likely to remain a mainstay of laboratory operations. It is recommended that larger plants invest in on-site IC systems which measure nutrient and also assist in monitoring scaling potential. Also for these sites a combination TOC/TNb systems offers results which are less ambiguous and faster than BOD, and have less environmental impact than COD.

Of the methods for monitoring microbial viability and vitality, the standout analytical tool in this field is ATP monitoring using 2nd Generation technology, which is now robust enough to supply quality data, quickly without scientific training. Oxygen uptake rates can also be useful however high quality well maintained DO probes and standardisation of temperature are key. Extracellular polymer concentrations are best monitored by measurement of turbidity after centrifugation.

From a practical standpoint monitoring microbial ecology of an MBR (via molecular technologies being used currently) is not likely to reveal much about the health of the process within a relevant timeframe. Neither is it a cost effective process control tool. Until these technologies progress and our knowledge of microbial ecology is more complete, simple light microscope observation will remain the best option.

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