Biomass active fraction evaluated by a direct method and respirometric techniques

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Abstract The knowledge of the active biomass amount and its characterisation is of primary importance for the management and for the design of wastewater treatment plants on the basis of the recently developed models. OUR curves obtained in aerobic exponential growth tests are proposed by several authors as an indirect method to estimate the active fraction. The aim of this work is the application of a direct method to measure the viable biomass based on flow cytometry techniques and the comparison with the active fraction obtained from respirometric tests. To assess the viable fraction of a biomass expressed in terms of COD concentration it is necessary to estimate the biovolume of viable bacteria and to know the specific carbon content per cell. For the investigated activated sludge samples, the bacterial biomass measured by FCM was 588 mgCOD L⁻¹ on average in a two-months period. This value of active fraction corresponds to 14% of particulate COD. Active biomass values measured with the respirometric approach were consistent but generally higher than those obtained by FCM.

Keywords Active biomass; activated sludge; flow cytometry; oxygen uptake rate; viability

Introduction
The activated sludge biomass in a MWWTP is normally quantified as concentration of volatile suspended solids (VSS). It is quite obvious that the mere measurement of VSS is not adequate to express the bacterial content of biomass and to know the relative activity. The quantification of the biomass active fraction is an important parameter in modelling activated sludge treatment plants, being required in simulation models as the UCT method (Ekama et al., 1984) or the Activated Sludge Model of IAWPRC Task Group (inter alia Henze et al., 1987; Henze, 1992; Gujer et al., 1995). The problems related to the direct measurement of active biomass and its use in plant design and in treatment process control give an additional interest for new characterisation approaches. In the literature, procedures for the biomass quantification are available for the bacteria present in lab culture or in different environments as soil (Bloem et al., 1995) or surface water (Blackburn et al., 1998). The most applied approach is to measure directly the bacterial biovolume and to convert it to bacterial biomass by multiplying the biovolume by the “weight of carbon per cell”, a conversion factor widely applied in microbiology (inter alia Fry, 1990; Loferer-Krösbacher et al., 1998). Epifluorescence microscope, electronic particle analysers, and scanning and transmission electron microscopy can be utilised as methods for the estimation of bacterial biovolume. The electronic particle analyser is not suitable to distinguish bacterial population from non-bacterial particles having the same size distribution. Bacterial cells can be easily distinguished from other particles using microscopic techniques coupled with fluorescent dyes staining. The standard procedure used to determine bacterial abundance and biovolume is the microscopic examination of cells stained with fluorochromes as 4’,6-diamidino-2-phenylindole (DAPI), or the recently developed dyes of the SYBR-Green I or SYTO family, that offer brighter fluorescence signals (Lebaron et al., 1998). Epifluorescence microscopy, coupled with charge-coupled device cameras and image-analysis software, has been successfully used for size measurement of small bacte-
ria (Viles and Sieracki, 1992). The size can be expressed by length and width that permit cell volume calculation applying appropriate formulas, which take into account the ellipsoidal shape of the rods or cocci (Blackburn et al., 1998; Sommaruga and Psenner, 1995). With respect to the time-consuming microscope examinations, the flow cytometry has recently been demonstrated to be a powerful tool. Flow cytometry (FCM) is a rapid method for measuring the optical properties of individual cells, such as light scattering (correlated with cell size) and fluorescence intensity, from which it is possible to discriminate between viable and dead bacteria (Barbesti et al., 2000). Viability can be assessed on the basis of membrane integrity, utilising SYBR-Green I (that enter in all the cells, both viable and dead) and Propidium Iodide (PI), which is the typical dye used to individuate permeabilised cells, considered as dead cells (Williams et al., 1998). FCM allows us to count in short time and in a reliable way more than 1,000 cells per second, being therefore more efficient than an epifluorescence microscope. It must be taken into account that most bacteria grow in flocs. Thus, for the measurement of bacteria abundance and biovolume estimation, a preventive dispersion of the aggregates, including bacteria, organic and inorganic debris, is needed. However all the approaches indicated above are generally expensive, complex to apply and, in general, only a little amount of sample can be examined. Simple procedures to indirectly evaluate heterotrophic active fractions in the activated sludge plants based on sludge age (Ekama et al., 1984) and respirometry (Kappeler and Gujer, 1992; Wentzel et al., 1998) are currently available.

In this paper the possibility to directly measure the viable fraction of particulate COD in activated sludge samples was investigated. A dual-staining technique using SYBR-Green I and Propidium Iodide was adopted for the direct estimation of viable bacteria abundance and of their biovolume combining FCM and epifluorescence microscope techniques. The results have been expressed in terms of COD, taking into account the specific content of carbon per cell. The values were then compared to those obtained from respirometric tests, according to the method proposed by Wentzel et al. (1998), based on OUR (Oxygen Uptake Rate) dynamics.

Materials and methods

Wastewater and activated sludge samples

Activated sludge samples were taken for a period of several months from the Trento Nord (Italy) activated sludge MWWTP having a capacity of 100,000 PE and a sludge age of about 17 days. The treatment line is composed of a primary settler followed by an activated sludge stage (oxidation tank and secondary settler). The organic load applied to the biological reactor was equal to about 0.24 kgCOD kg VSS⁻¹ d⁻¹ (average on the 2 months monitoring period) and the biomass concentration maintained in the oxidation tank was in the range 2.8–3.5 kgVSS m⁻³. Pre-settled wastewater was used for the respirometric tests in order to reproduce the influent into the activated sludge reactor.

The whole procedure

After sampling, the activated sludge and pre-settled wastewater were immediately delivered to the lab and respirometric tests (indirect measure of active biomass) were carried out. FCM analysis (direct measure of viable biomass) was performed on pre-treated samples. The flow-chart of the implemented procedure is synthesised in Figure 1.

FCM and microscopy examination

The main steps for the direct viable biomass measurement are the following (see Figure 1):

1. pre-treatment of the sample to perform a single-cell suspension according to the method described in Ziglio et al. (2002);
double staining with the DNA-specific fluorescent dyes SYBR-Green I and Propidium Iodide (Molecular Probes Inc., Eugene, Oregon) capable to discriminate viable and dead bacteria, emitting in green and red fluorescence respectively (Ziglio et al., 2002);

3. measurement of fluorescence signals by FCM (Bryte-HS flow cytometer, Bio-Rad Inc., Hercules, CA) and count of viable and dead bacteria;

4. measurement of viable cell biovolume by epifluorescence microscopy and image analysis. Forward-angle-light-scatter (FALS) acquired by FCM can also be used to estimate cell volume.

In order to obtain FALS distribution a few minutes are required. However scattering signals cannot be directly converted into biovolume. Therefore a calibration was performed examining a few stained samples under an epifluorescence microscope (time required 1–2 hours), which allows us to evaluate cell size and biovolume distribution utilising CCD camera and automated procedures. The relationship and the algorithm to convert FALS distribution into cell biovolume distribution is described in Ziglio et al. (2001).

Biovolume was converted into biomass on the basis of the carbon content per cell (Fry, 1990; Loferer-Krössbacher et al., 1998).

Respirometry
Temperature controlled 2 litre reactors were used as closed-respirometers. Aeration and mixing was guaranteed by compressed air and magnetic stirrer. Re-oxygenation of the mixed liquor must be avoided. Dissolved oxygen was monitored by an oxymeter (OXI 340, WTW GmbH, Weilheim, Germany) connected to a data acquisition system.

For evaluating the heterotrophic active fraction, two respirometric tests should be carried out: (1) with only raw or pre-settled wastewater; (2) with a known amount of biomass (ranging from 20 to 60 mL) taken from the oxidation tank and added to a 1.5 L of wastewater. The ratio S_0/X_0 (Substrate/Biomass) must be higher than 4 (Kappeler and Gujer, 1992). In order to inhibit the nitrification, about 10 mg allyl-thiourea L^{-1} was added at the beginning of the respirometric tests.

Lab analysis
All the chemical analyses (COD, VSS) were performed according to Standard Methods (APHA, 1995). The only exception was the soluble COD assessed through the soluble flocculated COD obtained according to Mamais et al. (1992). Readily biodegradable COD (RBCOD) was measured according to the respirometric method proposed by Ziglio et al. (2000).
Results and discussion

The two methodologies described in Figure 1 were applied and compared for estimating the active and viable biomass in activated sludge samples taken from Trento MWWTP.

Direct measurement of viable biomass

Viable bacteria counts, frequency distribution of the single-cell biovolume and specific carbon content are required to calculate the viable biomass. Table 1 summarises the results of the FCM analysis. In the two-months period of monitoring, the number of total viable counts in sludge samples averaged $1.6 \times 10^{12}$ cell L$^{-1}$ with little variation ($n = 6$, CV = 9.2%) and the total biovolume (calculated only for viable cells) was 693 mm$^3$ L$^{-1}$ on average (CV = 12.7%). Biovolume was converted into biomass on the basis of carbon content per unit of cell volume (Fry, 1990). Several authors relate that this conversion factor from biovolume into bacterial biomass changes depending on bacteria types and shows a large coefficient of variation when calculated experimentally. Fry (1990) recommends us to use a conversion factor of 310 fg µm$^{-3}$, specifically valid for epifluorescence microscopy applications. The carbon content of bacteria cells was assumed equal to 53% of the dry weight, based on the average bacteria composition of C$_7$H$_{10}$O$_3$N. The biomass dry weight was converted into COD multiplying by 1.48 mg COD per mg dry weight. In the monitoring period, in which the influent organic load was quite constant, the average viable fraction value resulting was equal to 13.7% of particulate COD.

The mean biovolume per cell (calculated dividing the total biovolume by the number of viable cells) was 0.43 µm$^3$ cell$^{-1}$ on average (CV = 11.3%). Cellular biovolumes equal or less than 0.08, 0.19 and 0.43 µm$^3$ cell$^{-1}$ were determined in correspondence of 25%, 50%, 75% of the total distribution respectively. Therefore only 25% of the bacterial population has a biovolume higher than the mean specific biovolume (0.43 µm$^3$ cell$^{-1}$). This fraction could be attributed either to large-size cells, bacteria aggregates not dispersed by pre-treatment steps, or cells in replication stage. Similar results for the cellular mean volume in the case of activated sludge were found by Münch and Pollard (1997).

The double staining with SYBR-Green I and PI allows us to detect viable bacteria, defined as cells having membrane integrity. The active biomass required in activated sludge models refers to the cells capable to exhibit respirometric or enzymatic activity. Activity is a more restrictive condition than membrane integrity and therefore cells exhibiting activity are a fraction of the viable cells. Previous investigations on Trento MWWTP demonstrated that enzymatically active bacteria, stained with fluorescein diacetate (FDA) were about 80% of total viable bacteria (Ziglio et al., 2002). The adoption of the double staining with SYBR-Green I and PI instead of FDA and PI was justified because the fluorescence signal is very bright and permits us to distinguish with high efficiency the bacteria signal from the instrument noise and sample background. Moreover the stained cells can be observed under epifluorescence microscope also after some hours, without any remarkable loss of fluorescence intensity, as in case of FDA staining.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Number of viable cells [cell counts L$^{-1}$]</th>
<th>Total biovolume [mm$^3$L$^{-1}$]</th>
<th>Mean biovolume [µm$^3$ cell$^{-1}$]</th>
<th>Total viable biomass [mgCOD L$^{-1}$]</th>
<th>Viable fraction [% of particulate COD]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$1.34 \times 10^{12}$</td>
<td>603</td>
<td>0.45</td>
<td>512</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>$1.65 \times 10^{12}$</td>
<td>693</td>
<td>0.42</td>
<td>588</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>$1.60 \times 10^{12}$</td>
<td>688</td>
<td>0.43</td>
<td>584</td>
<td>13.4</td>
</tr>
<tr>
<td>4</td>
<td>$1.72 \times 10^{12}$</td>
<td>602</td>
<td>0.35</td>
<td>511</td>
<td>13.0</td>
</tr>
<tr>
<td>5</td>
<td>$1.67 \times 10^{12}$</td>
<td>836</td>
<td>0.50</td>
<td>709</td>
<td>16.8</td>
</tr>
<tr>
<td>6</td>
<td>$1.76 \times 10^{12}$</td>
<td>739</td>
<td>0.42</td>
<td>628</td>
<td>14.9</td>
</tr>
<tr>
<td>Avg.</td>
<td>$1.62 \times 10^{12}$</td>
<td>693</td>
<td>0.43</td>
<td>588</td>
<td>13.7</td>
</tr>
</tbody>
</table>
The viable fraction was also evaluated on a few samples of pre-settled wastewater entering into the oxidation tank, having the following average characteristics: solids concentration of 124 mgSSV L\(^{-1}\); viable bacteria counts of \(3.2 \times 10^{10}\) cell L\(^{-1}\) and mean cellular biovolume of 0.57 µm\(^3\) cell\(^{-1}\). The latter value was higher than the one measured for activated sludge. Total viable biomass was 12 mg COD L\(^{-1}\) on average, corresponding to 7% of the particulate COD. Other authors found values ranging from 12–17% in primary settled wastewater (Spérandio et al., 2001) and in the range 2–7% (Wentzel et al., 1998), 7–15% (Kappeler and Gujer, 1992) and 2–13% (6.5% on average, Spérandio et al., 2001) in raw wastewater, expressed as percentage of total COD.

**Indirect measure of active biomass (respirometric tests)**

The active fraction in activated sludge can be evaluated by respirometric tests based on OUR. This technique was derived from the one proposed by Kappeler and Gujer (1992) and applied by Wentzel et al. (1998), who developed a method utilising two parallel respirometric tests: (1) on raw or pre-settled wastewater, (2) on wastewater inoculated by activated sludge biomass, with high Substrate/Biomass ratio (\(S_0/X_0\)), in order to reproduce the optimal organic loads for non-limiting bacterial growth. By plotting OUR values versus time the active fraction of the heterotrophic biomass and the relative specific maximum growth rate, \(\mu_{\text{max}}\), can be calculated.

**\(S_0/X_0\) ratio**

The influent wastewater of Trento MWWTP has a readily biodegradable COD (RBCOD) concentration of 68 mg COD L\(^{-1}\) (13% of the total influent COD). Sewage containing a low fraction of RBCOD were frequently reported by other authors. Spérandio and Etienne (2000) reported a mean value of 8.5% of total COD for French settled wastewater; Kappeler and Gujer (1992) report values between 7 and 11% for Swiss sewage.

An amount of about 20 to 60 mL of mixed liquor (2.8 g VSS L\(^{-1}\)) has to be usually added to 1.5 L of pre-settled wastewater to obtain clearly distinct OUR curves. In these conditions the \(S_0/X_0\) ratio resulted in a range of 0.4–1.2 mgCOD mgVSS\(^{-1}\) and thus was not sufficient to ensure the required ratio as proposed by Kappeler and Gujer (1992). Thus, when the respirometric tests were applied to influent wastewater with non-sufficient RBCOD concentration, further synthetic RBCOD (Sodium Acetate) had to be added (usually 50 mgCOD per litre) for reaching the required \(S_0/X_0\) ratio.

**OUR measurements**

The expression of oxygen respiration in time, without limitation, is:

\[
\text{OUR}(t) = \left(1 - \frac{X_0}{Y_H} \mu_{\text{max}} - b\right) \cdot X_0 \cdot e^{(\mu_{\text{max}} - b) \cdot t}
\]

(1)

and therefore the initial heterotrophic active biomass \((X_0)\) can be easily derived knowing the OUR(t) dynamic during an exponential growth phase. The \(\ln(\text{OUR})\) vs. time curve in exponential growth allows us to calculate \((\mu_{\text{max}} - b)\). Specific decay rate \((b)\) can be also estimated in batch respirometric tests (lasting 7–10 days under endogenous conditions) measuring the decrease of OUR or of viable bacteria counted by FCM (data not shown, the procedure is described in Ziglio et al., 2002). However, the mathematical expression used to calculate the active biomass is slightly influenced by the value of the decay rate. In the literature \(b\) values for heterotrophic bacteria range widely from 0.1 to 0.6 d\(^{-1}\) (see reviewed data in Avcioglu et al., 1998). This variability accounts for a 7% variation of the active biomass. In this work the endogenous decay rate \(b\) was assumed equal to 0.62 d\(^{-1}\) as indicated for the use in dynamic simulation models.
Exponential curves of OUR data were reported in logarithmic scale as shown in Figure 2. The linear interpolation of the data allows us to calculate the slope ($\mu_{\text{max}}-b$) and the intercept of the $y$-axis. The approach proposed by Wentzel et al. (1998) was applied to calculate the active biomass ($X_0$) in terms of mgCOD L$^{-1}$) in the two tests as follows:

$$X_0 = \frac{e^{(y-\text{intercept})}}{1-y} \cdot \frac{24}{(\text{slope}+b)}$$

where $Y$ is the yield coefficient for heterotrophic biomass, assumed equal to 0.67. Once the active biomass for both the tests was calculated, the active fraction of the activated sludge was obtained subtracting the $X_0$ value of pre-settled wastewater from the $X_0$ value of the mix (Figure 2). The active fraction calculation for a period of two months gave values consistently ranging from 27% to 78% (see the example in Figure 2). This high variability was found also for different additions of the same mixed liquor in the same wastewater. The wide range and the high values found seem unrealistic when compared to the most accredited theoretical values.

The critical factors which could affect the active biomass determination by respirometric techniques were analysed. Expression (2) used for active biomass calculation is mainly affected by the values of $y$-intercept and the slope of the interpolation line ($\mu_{\text{max}}-b$). We repeated the experiment adding different amounts of activated sludge mixed liquor (0 mL, 20 mL, 40 mL and 60 mL) to 1.5 litre of pre-settled wastewater. An amount of 700 mg COD per litre of wastewater (as Sodium Acetate) was added to each respirometer and OUR was measured. $S_0/X_0$ ratio ranged from 4 to 13. OUR dynamics for the 4 respirometers are shown in Figure 3A. The initial values of OUR (OUR$_0$) in the 4 different tests were linearly proportional to the increment of mixed liquor added (the average increment in the OUR$_0$...
was about 0.15 mgO₂ mL⁻¹ of mixed liquor added) as expected. Increasing the quantity of mixed liquor added, the slope clearly decreases. Further, the slope of each curve, i.e. the term \((\mu_{\text{max}} - b)\), plotted in y-logarithmic scale shows continuous variation in time. Considering (1) the following expression can be applied to calculate the parameter \((\mu_{\text{max}} - b)\):

\[
\frac{1}{\text{OUR}(t)} \frac{d\text{OUR}(t)}{dt} = \mu_{\text{max}} - b
\]

The central derivative of the discrete function was calculated and the derived function is shown in Figure 3B.

The maximum growth rate of wastewater was about 11.6 d⁻¹. The graph of \((\mu_{\text{max}} - b)\) vs. time in Figure 3B shows that the maximum growth rate of the respirometric tests running with mixed liquor addition was reached after about 5 hours. Therefore the value of the slope obtained in the first 3–4 hours could not be correct for active biomass calculation. For example the growth rate of Figure 2 was 4.07 d⁻¹, similar to the initial values found in Figure 3B. The maximum values were in the range of 6.6–7.9 d⁻¹, sufficiently similar for all the tests carried out with different dosages of mixed liquor (Figure 3B). The active biomass was calculated from expression (2), using the OUR₀ (i.e. the first point in the OUR curve) instead of the term exp(\(y\)-intercept) and the correct maximum growth rate, resulting in 1,470 mgCOD L⁻¹ on average (30.1% of the particulate COD in the activated sludge sample). The parameters calculated from the data of Figure 3 are summarised in Table 2.

![Figure 3](https://iwaponline.com/wst/article-pdf/46/1-2/371/476926/371.pdf)

**Figure 3** OUR dynamics of 4 tests carried out with pre-settled wastewater and different amounts of mixed liquor. (A) natural logarithm of OUR vs. time; (B) derivative calculated from Eq. (3) vs. time

**Table 2** Active biomass calculated with the maximum growth rate measured from Figure 3

<table>
<thead>
<tr>
<th>Amount of mixed liquor added</th>
<th>(\ln \text{OUR}_0)</th>
<th>((\mu_{\text{max}} - b))</th>
<th>Active biomass*</th>
<th>Active biomass in activated sludge sample**</th>
<th>Active fraction of particulate COD in activated sludge sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML mgO₂ L⁻¹ h⁻¹</td>
<td>d⁻¹</td>
<td>mgCOD L⁻¹</td>
<td>mgCOD L⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.54</td>
<td>11.6</td>
<td>6.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>1.56</td>
<td>7.3</td>
<td>27.1</td>
<td>1,544</td>
<td>31.6</td>
</tr>
<tr>
<td>40</td>
<td>2.02</td>
<td>6.8</td>
<td>45.6</td>
<td>1,465</td>
<td>30.0</td>
</tr>
<tr>
<td>60</td>
<td>2.31</td>
<td>6.6</td>
<td>62.5</td>
<td>1,400</td>
<td>28.6</td>
</tr>
</tbody>
</table>

* calculated applying Eq. (2) using the maximum growth rate extracted from Figure 3B and the \(\text{OUR}_0\) at the beginning of the respirometric test;  
** calculated taking into account the amount of activated sludge added and the dilution factor in 1.5 L of wastewater.
In our MWWTP, correct estimation of active biomass through respirometric tests, cannot be obtained with the first slope in the OUR-diagram. We can also suppose that the maximum slope found in our case (Figure 3B) is not the potential maximum growth rate for the bacteria population. For this activated sludge sample the bacterial biomass measured by FCM was 588 mgCOD L\(^{-1}\). If we put this value as \(X_0\) in the Eq. (2) and we repeat our calculation, the resulting \(\mu_{\text{max}}\) is about 11 d\(^{-1}\), that is, the value of the maximum growth rate shown by the pre-settled wastewater.

**Conclusions**

A dual-staining technique using SYBR-Green I and Propidium Iodide was adopted for the direct estimation of the abundance of viable bacteria and of their biomass, combining FCM and EM techniques. The active biomass has been expressed in terms of COD, taking into account the specific content of carbon per cell, and then compared to the one obtained from respirometric tests. The application of the direct and indirect methods has drawn the following conclusions.

- **Active biomass measurements by FCM show little variation during time and a good reproducibility, when evaluated on triplicate samples. On average the active fraction was equal to 14% of the particulate COD in a two-months period. This value is lower than the expected value on the basis of the theoretical approach, but in agreement with the ones obtained by Münch and Pollard (1997). However the conversion of biovolume into biomass is influenced by the specific content of carbon per cell, assumed in this work equal to 310 fg/cell, as suggested by Fry (1990).**
- **The respirometric method proposed by Wentzel et al. (1998) has been modified, because of the variability of the first slope in the OUR-diagram, which does not represent the maximum growth rate in our full-scale activated sludge WWTP. The active biomass measured with the respirometric approach was consistent but higher than those obtained by FCM. In order to obtain the FCM values, the maximum growth rate should be in the order of 11 d\(^{-1}\), higher than the activated sludge value, but similar to the pre-settled wastewater one.**
- **Further investigations are required to understand the initial behaviour in the OUR dynamic (substrate storage? How many bacteria are capable of showing an exponential growth phase?) to confirm these observations in other MWWTP plants with different COD composition, loads and sludge ages.**

**Acknowledgement**

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