3

RESPIROMETRY

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3.1 INTRODUCTION

The objective of this chapter is to provide practical guidelines for the assessment of the respiration rate of biomass. The approach will be practically oriented and method-driven. However, some biochemical background on respiration will be provided in order to understand how respiration is related to microbial substrate utilization and growth. We will explain that respiration can be assessed in terms of the uptake rate of a terminal electron acceptor, such as molecular oxygen or nitrate, or, in the case of anaerobic respiration, in terms of the production rate of methane or sulphide. The measurement of the consumption (or production) rate, i.e. respirometry, will be explained following various measuring principles, and we will provide some practical recommendations. The focus will be on laboratory tests using samples of biomass and wastewater. However, in principle most measuring principles can be automated, or have already been automated in commercial respirometers, to measure respiration rate automatically or even in-line at a wastewater treatment plant. In-line measurement of respiration rate, however, is out of the scope of this textbook. One specific method, off-gas analyses, provides an inherent way to assess the respiration rate in a pilot-scale or full-scale wastewater treatment plant. This method is described in Chapter 4.

The information that can be extracted from respirometric measurements can be divided into two types: direct and indirect (Spanjers et al., 1998). Direct information, such as the aerobic respiration rate or specific methanogenic activity, provides information on the actual activity of the biomass, and can be used for example to record respiromgrams (time series of respiration rates) in the lab. Indirect information refers to variables that are deduced from respirometric measurements, such as microorganism concentration, substrate concentration and kinetic parameters. In this case respirometric measurements are used as input to simple arithmetic calculations or even model fitting. Chapter 5 will describe how data such as respirometric data can be used in model fitting to assess deduced variables and parameters.

Because the objective of this chapter is to provide only practical guidelines for the assessment of the respiration rate, only a basic explanation of the biochemical background will be provided, and the reader is referred to the literature on biochemistry (Alberts et al., 2002; Nelson and Cox, 2008). As the practical guidelines focus on respirometric methods that can easily be carried out in most laboratories, no rigorous discussion of all the respirometric measuring principles will be given, and we
refer to Spanjers et al. (1998) for a more complete description of the concepts. For a discussion of the use of direct and indirect respirometric information in control of the activated sludge process the reader may consult Copp et al. (2002).

### 3.1.1 Basics of respiration

In biochemical terms, microbial respiration is the adenosine triphosphate (ATP)-generating metabolic process in which either organic or inorganic compounds serve as the electron donor and inorganic compounds serve as the terminal electron acceptor (e.g. oxygen, nitrate, sulphate). The universal energy carrier ATP is generated as electrons removed from the electron donor are transferred along the electron transport chain from one metabolic carrier to the next and, eventually, to the terminal electron acceptor. In this way, microorganisms convert the energy of intramolecular bonds in the electron donor to the high-energy phosphate bonds of ATP (catabolism). The energy is then used to synthesize the various molecular components required for cell growth (anabolism), maintenance and reproduction.

During the process of respiration the electron donor is converted to its oxidized form and the electron acceptor is converted to its reduced form. In the case of a carbonaceous donor (organic compounds), the oxidized form is carbon dioxide. If the electron acceptor is molecular oxygen then its reduced form is water. The conversion of a carbonaceous donor with oxygen as the electron acceptor is carried out by heterotrophic bacteria.

Inorganic donors that are converted to their oxidized form by aerobic microorganisms, where oxygen serves as the terminal electron acceptor, include ammonium and nitrite, ferrous (divalent iron) and sulphide, and the conversions are carried out by nitrifiers (ammonia and nitrite-oxidizing bacteria), iron-oxidizing bacteria and sulphide-oxidizing bacteria, respectively. In this case CO₂ forms the source of carbon, and the organisms are called autotrophs. Non-aerobic microorganisms use inorganic compounds other than oxygen such as nitrite, nitrate, sulphate and carbon dioxide as the terminal electron acceptor. In these cases we are talking about anoxic (nitrite, nitrate) and anaerobic processes (CO₂, sulphate). Note that in wastewater treatment various respiration processes may take place simultaneously where different microorganisms use diverse substrates and terminal e-acceptors or compete for the same substrates and terminal electron acceptors.

Figure 3.1 shows a schematic overview of some examples of metabolic conversions. Note that both the electron donor and the terminal electron acceptor may be considered as substrate, like many other components that enter the metabolic pathways. In respirometry, respiration is generally considered as the consumption of O₂, NO₂⁻ or NO₃⁻ or (in anaerobic respirometry) the production of CH₄. In general terms, the metabolic conversions involved in respiration are catabolic reactions, and several gaseous compounds consumed or produced during these reactions can be used to assess key metabolic conversions. Note, however, that in principle other substances may also be considered, such as the consumption of NH₄⁺, HS⁻ or S²⁻, or SO₄²⁻, or the production of N₂. Other products that are not included in the figure but that are also linked to respiration include H⁺ and heat and the associated methods are titrimetry and calorimetry, respectively. However, these are out of the scope of this chapter.

![Figure 3.1 Schematic overview of some examples of metabolic conversions.](image)

Figure 3.1 Schematic overview of some examples of metabolic conversions. $e^-$ denotes the electron that is transferred from the electron donor to the terminal electron acceptor. [CHO] denotes any carbohydrate. The coloured substances are generally used as measured variables in respirometry.

Because the energy generated during the process of microbial respiration is used for cell growth and maintenance functions, such as reproduction, cell mobility, osmotic activity, etc., the respiration rate is linked to the rate of these processes. However, it is difficult to differentiate between these two processes. As an example, consider the aerobic respiration by heterotrophic microorganisms that use carbonaceous...
(organic) substrate as the electron donor and oxygen as the terminal electron acceptor. Only a portion (1-Y) of the consumed organic substrate is oxidised to provide energy for cell growth and maintenance. The remainder, typically half (on a weight/weight basis) of the substrate molecules (the yield Y) is reorganised into new cell mass. Hence the oxygen consumption rate is linked to the biomass growth through the yield. In anaerobic respiration of hydrogenotrophic methanogens, where H₂ substrate is used as the electron donor and CO₂ as the electron acceptor, only a small portion of Y of the substrate is rearranged into biomass, while the largest part is oxidized to produce CH₄.

In activated sludge, carbonaceous substrate removal is not the only oxygen-consuming process. In addition to the oxygen consumption by heterotrophic biomass, there are some other biological processes that may contribute to the respiration of activated sludge, such as the oxidation of inorganic compounds by nitrifiers and other bacteria, and specific microbial oxidation reactions catalysed by oxidases and mono-oxygenases. Nitrifying bacteria incorporate only a minor part of the substrate ammonia into new biomass while most of the substrate (ammonium) is oxidised for energy production. These autotrophic bacteria use dissolved carbon dioxide as a carbon source for new biomass. In comparison to heterotrophic biomass, nitrifiers need more oxygen for their growth. Nitrification occurs in two steps: the oxidation of ammonia to nitrite and the oxidation of nitrite to nitrate. Like nitrifiers, the autotrophic sulphur-oxidizing bacteria and iron-oxidizing bacteria utilise inorganic compounds instead of organic matter to obtain energy and use carbon dioxide or carbonate as a carbon source. Sulphur-oxidizing bacteria are able to oxidise hydrogen sulphide (or other reduced sulphur compounds) to sulphuric acid. Iron-oxidizing bacteria oxidise inorganic ferrous iron to the ferric form to obtain energy. In addition to bacteria, protozoa and other predating higher organisms are present in the activated sludge, and they also consume oxygen. Finally, some inorganic electron donors such as ferrous iron and sulphide can be chemically oxidised, also utilising oxygen.

All the above-mentioned oxygen-consuming processes contribute to the total respiration rate of the activated sludge. Respirometry is usually intended to measure only biological oxygen consumption and sometimes it is attempted to distinguish between different biological processes such as heterotrophic oxidation and nitrification. However, in many cases it is difficult to distinguish between specific microbial processes and to identify chemical oxygen consumption.

**3.1.2 Basics of respirometry**

Respirometry is generally defined as the measurement and interpretation of the rate of biological consumption of an inorganic electron acceptor under well-defined experimental conditions. In principle, all the substances depicted in the ‘substrate-oxidized form’ highlighted in Figure 3.1 can serve as the measured variable. An exception is anaerobic respirometry where generally the production rate of the ultimate reduced product methane is measured. This is because during anaerobic degradation many intermediates are involved and it is impracticable to measure the consumption rate of these intermediate substrates. Moreover, methanogenesis is generally not the rate-limiting step; hence, the methane production rate reflects the rate-limiting process (mostly hydrolysis in the case of a complex substrate).

Note that in principle one may also measure the consumption rate of the electron donor, such as [CHO], NH₄⁺ and H₂. However, this is generally not considered to be respirometry, also because electron donor substances such as [CHO] and NH₄⁺ may also be consumed by processes not related to energy generation, for example the uptake in biomass, and hence not clearly related to energy generation. Finally, measurement of CO₂ production may be considered respirometry because CO₂ production is related to energy generation (Figure 3.2). However, because CO₂ in the gas phase is associated with the carbonate system, extra measurements will be needed to measure the pH and bicarbonate concentration in the liquid phase.
Respirometry always involves some technique for assessing the rate at which the biomass takes up the electron acceptor (such as O<sub>2</sub> and NO<sub>3</sub>-) from the liquid or produces its reduced form (such as CH<sub>4</sub>); see Figure 3.2. For electron acceptors such as O<sub>2</sub> and NO<sub>3</sub> this is generally based on measuring the concentration of the electron acceptor in the liquid phase and solving its mass balance to derive the respiration rate. If the oxygen consumption is measured and a gas phase is present, one has to consider the mass balance of oxygen in the gas phase as well. Similarly, if measuring the production rate of methane, the mass balance of methane in both the liquid phase and the gas phase has to be considered.

For aerobic respirometry i.e. assessing the rate at which biomass takes up O<sub>2</sub>, Spanjers et al., (1998) presented a classification of respirometric principles that was based on two simple criteria: the first one being the location of the oxygen measurement, liquid or gas phase; and the second being the state of the gas and liquid phases, both either flowing or static. It was found that the majority of the proposed respirometric devices could be put into one of the eight classes created by this structure. Moreover, for each of the classes, examples of implementations were found in the literature.

### 3.2 GENERAL METHODOLOGY OF RESPIROMETRY

#### 3.2.1 Basics of respirometric methodology

The respiration rate is usually measured with a respirometer. Respirometers range from a simple, manually operated bottle equipped with a sensor to complicated instruments that operate fully automatically. In some cases the bioreactor of the treatment plant itself can serve as a respirometer. Except for the latter, a feature common to all respirometers is a reactor, separated from the bioreactor, where different components (biomass, substrate, etc.) are brought together. The operation of all respirometers involves some technique for assessing the rate at which the biomass takes up a component from the liquid or produces a component (Figure 3.2). Many techniques have been developed in the past. However, Spanjers et al. (1998) found that all measuring techniques for the respiration rate can be classified into only eight basic principles according to two criteria: (1) the phase where the concentration is measured (gas or liquid, G and L, respectively) and (2) whether or not there is input and output of liquid and gas (flowing or static, F and S, respectively). The operation of all existing respirometers can be explained in terms of these criteria. Figure 3.3 shows a generic scheme for a respirometer. Note that the gas phase also includes bubbles dispersed in the liquid phase. In the subsequent sections, the principles will be discussed according to the above criteria. We will not discuss the usefulness of the different measuring techniques, because we believe that any technique has its merits, depending on the specific application, provided that the correct measuring conditions are satisfied.

![Figure 3.3 Generic scheme of a respirometer.](image)

#### 3.2.2 Generalized principles: beyond oxygen

##### 3.2.2.1 Principles based on measuring in the liquid phase

The majority of the techniques based on measurement in the liquid phase use a specific electrode or sensor. A reliable respiration rate measurement is only possible if the sensor is correctly calibrated and if a number of environmental variables, such as temperature and pressure, is accounted for. Sensors also have a response time that must be accounted for in some respirometric setups.

Respirometers that are based on measuring dissolved oxygen (DO) concentration in the liquid phase use a DO mass balance over the liquid phase. Consider a system consisting of a liquid phase, containing biomass, and a gas phase both being ideally mixed and having an input and output (Figure 3.4). It is assumed that the DO concentration in the liquid phase can be measured. The DO mass balance over the liquid phase is:

\[
\frac{d(V_L \cdot S_{O_2})}{dt} = Q_{in} \cdot S_{O_2,in} - Q_{out} \cdot S_{O_2} + V_L \cdot k_La \cdot (S_{O_2}^* - S_{O_2}) - V_L \cdot r_{D2}
\]

Eq. 3.1

Where, \( S_{O_2} \) is the DO concentration in the liquid phase (mg L<sup>-1</sup>), \( S_{O_2}^* \) is the saturation DO concentration in the liquid phase (mg L<sup>-1</sup>), \( S_{O_2,in} \) is the DO concentration
in the liquid phase entering the system (mg L\(^{-1}\)), \(kLa\) is the oxygen mass transfer coefficient, based on liquid volume (h\(^{-1}\)), \(Qin\) is the flow rate of the liquid entering the system (L h\(^{-1}\)), \(Qout\) is the flow rate of the liquid leaving the system (L h\(^{-1}\)), \(rO_2\) is the respiration rate of the biomass in the liquid, (mg L\(^{-1}\) h\(^{-1}\)), and \(V_L\) is the volume of the liquid phase (L).

\[
\Delta S_{O2}/\Delta t = -r_{O2}.
\]
Typical of this principle is that the DO may become exhausted after some time so that for continued measurement of \(rO_2\) reaeration it is necessary to bring the DO concentration back to a higher level. DO and substrate limit the respiration when their concentrations become too low, causing a non-linear DO decrease complicating the assessment of the differential term. Note that in Figure 3.5 there is a gas phase. However, it is assumed there is no mass transfer from the gas phase into the liquid phase. In practice, in order to prevent the input of oxygen into the liquid, the gas phase may be absent. The procedure for the determination of \(rO_2\) according to standard methods (APHA \textit{et al.}, 2012) is based on this principle.

\[
\frac{dS_{O2}}{dt} = kLa \cdot (S_{O2} - S_{O2}^c) - r_{O2}
\]
Eq. 3.3

\[
\Delta S_{O2}/\Delta t = -r_{O2}.
\]
the aeration coefficients can be updated relatively easily. This respirometric principle allows the measurement of \( r_{O_2} \) at a nearly constant DO concentration, thereby eliminating the dependency of \( r_{O_2} \) on the DO concentration (provided DO >> 0 mg L\(^{-1}\)). Note that, whereas Figure 3.6 shows an input and an output in the gas phase, there is no gas flow term in Eq. 3.3. There is no need to consider gas flow terms provided \( S'_{O_2} \) is known or determined.

**Figure 3.6** The liquid phase principle, flowing gas, static liquid (LFS).

- **Static gas, flowing liquid (LSF)**

  Repetitive aeration or estimation of oxygen transfer coefficients, as with the above principles, can be avoided when liquid with a high enough input DO concentration flows continuously through a closed completely mixed cell without the gas phase (Figure 3.7). The liquid flow terms now have to be included in the mass balance (Eq. 3.4):

  \[
  \frac{dS_{O_2}}{dt} = \frac{Q_{m}}{V_{L}} S_{O_2,in} - \frac{Q_{out}}{V_{L}} S_{O_2} - r_{O_2} \tag{Eq. 3.4}
  \]

  **Figure 3.7** The liquid phase principle, static (no) gas, flowing liquid (LSF).

  Both DO concentrations, \( S_{O_2,in} \) and \( S_{O_2} \), must be measured to allow calculation of \( r_{O_2} \). In a respirometer \( Q_{m} \)

and \( V_{L} \) are instrument constants and are, therefore, assumed to be known or calibrated. This principle is in fact the continuous counterpart of the one explained in Eq. 3.2, and it is as such also sensitive to the effect of substrate and DO limitation. However, the effect of limiting substrate can be eliminated by the continuous supply of substrate (wastewater) and DO to the respiration cell.

- **Flowing gas, flowing liquid (LFF)**

  Without the above simplifications the full mass balance (Eq. 3.1) holds for the principle depicted in Figure 3.4. To obtain respiration rate measurements with this principle, a combination of the approaches mentioned for the above simplified principles is required. For instance, the flow rates and the inlet oxygen concentrations must be measured, while the coefficients \( k_{La} \) and \( S'_{O_2} \) must be assessed, e.g. by estimating these from the dynamics of the DO concentration.

### 3.2.2.2 Principles based on measuring in the gas phase

Respirometric techniques based on measuring gaseous oxygen always deal with two phases: a liquid phase containing the respiring biomass and a gas phase where the oxygen measurement takes place. The main reason for measuring in the gas phase is to overcome difficulties associated with interfering contaminants common in the liquid phase (e.g. the formation of biomass film on the sensor). Gaseous oxygen is measured by physical methods such as the paramagnetic method, or gasometric methods.

Gasometric methods measure changes in the concentration of gaseous oxygen. According to the ideal gas law \( P \cdot V = n \cdot R \cdot T \), these can be derived from changes in the pressure (if volume is kept constant, the manometric method) or changes in the volume (if pressure is kept constant, the volumetric method). These methods are typically applied to closed measuring systems (no input and output streams), which may provoke a need for reaerations and thus temporary interruption of the measurements. This limits the possibility for continued monitoring of the respiration rate. However, interruptions because of reaerations are not needed if the consumed oxygen is replenished at a known rate, e.g. by supplying pure oxygen from a reservoir or by using electrolysis. The rate at which oxygen is supplied is then equivalent to the biological respiration rate (assuming infinitely fast mass transfer to the liquid). Because carbon dioxide is released from the
liquid phase as a result of the biological activity, this gas has to be removed from the gas phase in order to avoid interference with the oxygen measurement. In practice this is done by using alkali to chemically absorb the carbon dioxide produced.

Respirometric principles based on measuring gaseous oxygen also use oxygen mass balances to derive the respiration rate. However, in addition to the mass balance in the liquid phase (Eq. 3.1), a balance in the (ideally mixed) gas phase must be considered (Figure 3.8):

$$\frac{d}{dt}(V_o \cdot C_{O_2}) = F_{in} \cdot C_{O_2,in} - F_{out} \cdot C_{O_2} - V_o \cdot k_{La} \cdot (S_{O_2}^* - S_{O_2})$$

Eq. 3.5

Where, $C_{O_2}$ is the $O_2$ concentration in the gas phase (mg L$^{-1}$), $C_{O_2,in}$ is the $O_2$ concentration in the gas entering the system (mg L$^{-1}$), $F_{in}$ is the flow rate of the gas entering the system (L h$^{-1}$), $F_{out}$ is the flow rate of the gas leaving the system (L h$^{-1}$), and $V_o$ is the volume of the gas phase (L).

Figure 3.8 The gas phase principle, flowing gas, flowing liquid (GFF).

The term $V_o \cdot k_{La} \cdot (S_{O_2}^* - S_{O_2})$ represents the mass transfer rate of oxygen from the gas phase to the liquid phase, and it is the connection between the two phases. From mass balances (Eq. 3.1 and Eq. 3.5) it follows that, in order to calculate $r_{O_2}$, $C_{O_2}$ must be measured (directly or using the gas law, see above) and knowledge of $S_{O_2}$ is required. However, $S_{O_2}$ is not measured in the gas phase principles. In these respirometric principles it is assumed that the oxygen concentrations in the gas and liquid phases are in equilibrium, i.e. mass transfer is sufficiently fast ($k_{La} \rightarrow \infty$), so that $S_{O_2} \approx S_{O_2}^*$. Since, by definition, the saturation DO concentration is proportional to the $O_2$ concentration in the gas phase:

$$S_{O_2}^* = H \cdot C_{O_2}$$

Eq. 3.6

it is reasonable to state that:

$$S_{O_2} = H \cdot C_{O_2}$$

Eq. 3.7

and that:

$$\frac{dS_{O_2}}{dt} = H \cdot \frac{dC_{O_2}}{dt}$$

Eq. 3.8

Hence, the measurement in the gas phase is a good representation of the condition in the liquid phase, provided the proportionality (Henry) constant $H$ is known, e.g. from calibration or tables, and the mass transfer coefficient is high. The validity of this equilibrium assumption should be critically evaluated.

- **Static gas, static liquid (GSS)**

The simplest gas phase technique for measuring the respiration rate is based on a static liquid phase and a static gas phase, i.e. no input or output (Figure 3.9). In addition to the DO mass balance in the liquid phase, an oxygen mass balance in the gas phase must be considered:

$$\frac{dS_{O_2}}{dt} = k_{La} \cdot (S_{O_2}^* - S_{O_2}) - r_{O_2}$$

Eq. 3.9

$$\frac{d(V_o \cdot C_{O_2})}{dt} = - V_o \cdot k_{La} \cdot (S_{O_2}^* - S_{O_2})$$

Eq. 3.10

Hence, in order to calculate $r_{O_2}$, the change of the oxygen concentration in the gas phase, $dC_{O_2}/dt$, must be measured and knowledge of $dS_{O_2}/dt$ is required (Eq. 3.9). It is possible to measure $dC_{O_2}/dt$ by using an oxygen sensor. If a gasometric method is used, $dC_{O_2}/dt$ is related to the change in volume or the change in pressure (Eq. 3.10).

Figure 3.9 The gas phase principle, static gas, static liquid (GSS).
With this principle, the same restriction as with the simplest DO-based principle exists: when the oxygen becomes exhausted it must be replenished by, for instance, venting the gas phase in order to continue the measurement of \( r_{O_2} \).

- **Flowing gas, static liquid (GFS)**

Another technique is based on a flowing gas phase, i.e. the biomass is continuously aerated with air (or pure oxygen) so that the presence of sufficient oxygen is assured (Figure 3.10). In comparison to Eq. 3.10, two transport terms must be included in the mass balance on the gas phase:

\[
\frac{dS_{O_2}}{dt} = k_{La} \cdot (S'_{O_2} - S_{O_2}) - r_{O_2} \quad \text{Eq. 3.11}
\]

\[
\frac{d(V_{G} \cdot C_{O_2})}{dt} = F_{in} \cdot C_{O_2,in} - F_{out} \cdot C_{O_2} - V_{L} \cdot k_{La} \cdot (S'_{O_2} - S_{O_2})
\]

**Figure 3.10** The gas phase principle, flowing gas, static liquid (GFS).

In order to allow the calculation of \( r_{O_2} \), the gas flow rates, \( F_{in} \) and \( F_{out} \), and the oxygen concentrations in the input and output streams, \( C_{O_2,in} \) and \( C_{O_2} \), must be known in addition to the variables of the previous technique. Of these, usually \( C_{O} \) is measured and the others are set or known. A gasometric method is not evident here, and the measurement of \( C_{O} \) is done for example with the paramagnetic method.

- **Static gas, flowing liquid (GSF)**

Implementations of the gas phase principle with static gas and flowing liquid (Figure 3.11) have not been found in literature or in practice so far.

**Figure 3.11** The gas phase principle, static gas, flowing liquid (GSF).

- **Flowing gas, flowing liquid (GFF)**

The gas phase principle can also be applied to a full-scale bioreactor. In this case there are liquid input and output streams for the reactor, and transport terms must be added to the mass balance in the liquid phase (Eq. 3.1). The assumption on proportionality between \( C_{O_2} \) and \( S_{O_2} \) (Eq. 3.7) becomes more critical because, in addition, the liquid outflow term also depends on it. Additional measurement of dissolved oxygen may then be useful for a correct assessment of the respiration rate. The technique then would no longer be a pure gas phase principle. Note, however, that in general combining \( L \) and \( G \) principles may lead to more reliable respiration rate measurements.

Table 3.1 summarises the eight measuring principles. The first column contains the names of the mass balance terms, and the second column the mathematical equivalents. The succeeding columns list the respirometric principles, the first four being the liquid-phase principles, and the others being the gas-phase principles. The mass balances for each principle are formed by multiplying the mathematical terms with the coefficients in the column of the appropriate principle and summing them up.
### 3.3 EQUIPMENT

#### 3.3.1 Equipment for anaerobic respirometry

To carry out an anaerobic respirometric test there are two requirements. Firstly, a setup is needed in which anaerobic respiration takes place. This can be a small bottle or larger reactor. In this bottle or reactor a substrate, for example, primary sludge or starch, and an inoculum with the consortia required for anaerobic respiration are combined. Secondly, a system to measure the methane production is required. To quantify anaerobic respiration, the flow of electrons has to be determined. Neither the consumption of substrate nor the consumption of an electron acceptor can be measured directly and therefore the final products of anaerobic respiration, H2 and methane, are determined in anaerobic respirometry.

##### 3.3.1.1 Biogas composition

Methane leaves the bottle or reactor via the biogas. Besides methane, biogas also contains CO2, H2S and traces of other compounds. Thus, in order to quantify the methane flow, both the biogas flow and the biogas composition need to be known. To this end, the composition of biogas can be either measured or adapted. Adaptation of the biogas means removing all gas other than methane prior to flow quantification.

- **Measuring the biogas composition and correcting the measured flow**

Measuring the biogas composition can be done with gas chromatography. However, there are also cheaper and easier methods, for example, the apparatus shown in Figure 3.12.

![Figure 3.12 An inexpensive and simple way for determining the methane concentration in biogas.](image-url)
EXPERIMENTAL METHODS IN WASTEWATER TREATMENT

3.3.1.2 Measuring the gas flow

There is a large variety of ways to measure gas production but in lab-scale anaerobic respirometry it is usually limited to manometric or volumetric methods.

- **Manometric methods**
  Manometric methods are based upon measuring pressure increase in the head space of a reaction vessel. As biogas is produced, the pressure in the headspace increases. However, high headspace pressure can result in increased CO₂ solubility, which may significantly disturb microbial activity (Theodorou et al., 1994). Therefore, the pressure needs to be released periodically to prevent it from becoming too high (pressure release). Generally, an upper limit of 1.4 bar is applied. One must also make sure that the reaction vessel is designed to withstand the pressure. When the pressure is not released automatically, this method requires labour during the experiment. Inappropriate operation can lead to explosion of the reaction vessel. It is strongly recommended to always use safety goggles when working with the manometric method. In addition, the initial pressure measurement and the measurements after draining should also consider the temperature effect on gas pressure and water vapour pressure, i.e. an equilibrium condition shall be reached in the reaction vessels before measurement. To calculate the gas production, the pressure increases between two pressure drains are summed and with the ideal gas law the amount of produced moles of biogas is calculated from this total increase in pressure. The composition of the biogas needs to be measured as well if there is no CO₂ and H₂S scrubbing.

- **Volumetric methods**
  A classical and robust volumetric method is to use Mariotte’s bottle (McCarthy, 1934), where the gas is introduced into a bottle with an outlet for the liquid and it displaces the liquid (Figure 3.14). The weight or volume of the displaced liquid indicates the volume of gas that is produced. When the liquid is an alkaline solution, CO₂ or H₂S is scrubbed in situ and the weight of displaced liquid will indicate the volume of methane. A disadvantage is that the flask needs to be refilled periodically, which disrupts the pressure control. If the displaced liquid is measured with a balance connected to a computer, the gas production can be measured in real time.
3.3.2 Equipment for aerobic and anoxic respirometry

Similar to anaerobic respirometry, a setup is needed in which aerobic and anoxic respiration takes place. This basically consists of a stirred bottle or reactor where biomass under aerobic or anoxic conditions and wastewater, or a specific substrate, are combined. In addition, an arrangement is required to measure the uptake of the terminal electron acceptor, i.e. oxygen, nitrite or nitrate. Data handling may be manually, as is mostly the case in BOD measurements, or completely automated, for example if the measured data is to be converted to respiration rates with a high measuring frequency. In a number of sophisticated (including commercial) respirometers the operation of the equipment is so complicated that it requires an automated control system.

### 3.3.2.1 Reactor

The reactor is usually a vessel with a volume ranging from a few 100 mL to several litres. Depending on the application, laboratory or field, the material may be glass or plastic, and is often transparent in order to enable inspection of the content. Depending on the measuring principle (Section 3.3.1.2), the vessel is completely sealed to prevent the exchange of oxygen with the gas phase, or open to allow the transfer of oxygen from the gas phase. Open vessels may also be equipped with aeration equipment (e.g. a sparger) to enhance oxygen transfer. In some cases the vessel may be operated in both open mode (for aeration) and closed mode (for measuring oxygen uptake). In all cases, the vessel is completely mixed, with a magnetic bar, an impeller, a pump, or by aeration. In the laboratory the vessel may be thermostated, often using a double wall for cooling/heating or just a heating element if the temperature is maintained above ambient temperature. Depending on the operation principle (flowing liquid, flowing gas), the reactor may have several inlet and outlet ports, and one or more apertures to accommodate (a) sensor(s). Supplementary equipment may include valves, pumps (for biomass, wastewater, substrate, air, and gas), a mixing tank, substrate container, oxygen container, NO₃ supply container, sample pre-treatment unit (sieve, filter), oxygen generator, etc.

### 3.3.2.2 Measuring arrangement

In many cases the measuring arrangement consists of a sensor (i.e. a probe with an associated meter whether or
not connected to a data acquisition system) to measure the concentration of the electron acceptor, i.e. oxygen, nitrite or nitrate. Oxygen may be measured directly in the liquid phase with a galvanic, polarographic or optical dissolved oxygen probe. In simple laboratory tests, especially a BOD test, dissolved oxygen may be measured with a titrimetric or photometric method (Section 3.4.2). The oxygen concentration in the gas phase may be directly measured with a paramagnetic oxygen analyser. However, changes in oxygen concentration may be measured by means of a pressure sensor or gas volume displacement sensor. Nitrate and nitrite concentrations in the liquid phase can be measured with an ion-selective or UV-spectrophotometric sensor (Rieger et al., 2008).

Sensors may have slow response times, and it is important to ensure that the sensor is fast enough to follow the kinetics of the biochemical process. As a rule, the sensor must be 10 times faster than the measured reaction rate.

### 3.3.2.3 Practical implementation

Many practical implementations have been described in the literature and a number of them have been introduced onto the market. As explained in Section 3.3.1, all the measuring techniques for the respiration rate can be classified into only eight basic principles and the operation of all the existing respirometers can be explained in terms of this classification and the corresponding mass balances. However, only a limited number of respirometers have been applied in considerable numbers in research and practice, or even commercial production. In what follows we describe some respirometers in terms of their basic principle and technical implementation. However, it should be emphasized that by no means should this be understood as a recommendation for a specific method. The choice of a certain measuring principle, its technical implementation, or commercial manifestation depend on the measurement purpose, skill of the user and available budget.

- **Liquid phase, static gas, static liquid (LSS) principle**
  The LSS principle can be considered as the simplest respirometric principle because the absence of flowing liquid and gas implies that no supplementary materials, such as pumps or aeration equipment, are needed. The BOD test (Section 3.4.2) is an example of the application of this principle. Figure 3.16 shows an example of a BOD bottle used in a test where DO is only measured in the beginning and at the end of the test, and an example of a BOD bottle with continuous measurement of the oxygen uptake by means of a pressure sensor. The latter allows for the assessment of the ultimate BOD and the first order oxygen uptake rate coefficient (Section 3.4.2).

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*Figure 3.16* A BOD bottle for the classical BOD test (left) and bottles for the continuous measurement of oxygen uptake (right) (photos: Wheaton and VELP Scientifica).
However, the LSS principle has also been implemented in a semi-continuous version to measure the respiration rate of biomass semi-continuously, both in the lab and in the field. Because of the much higher biomass concentration than used in a typical BOD test, the DO concentration drops due to respiration within a few minutes from a near saturated concentration to a limiting concentration. The respiration rate is then calculated from the slope of the DO concentration decline. To allow repeated measurement of the respiration rate the biomass is reaerated after each measurement, which yields a typical saw-tooth DO profile (Figure 3.17). In this example the time between the on/off periods of aeration is constant. Other respirometers make the aeration switch on and off dependent on the actual DO concentration, e.g. between 4 and 6 mg O₂ L⁻¹. These upper and lower DO limits should be defined carefully; they determine the frequency of respiration rate date and their accuracy. Indeed, when the respiration rate is low, it may take a long time to lower the DO concentration from the upper to the lower limit, whereas too short declines make the calculation of the respiration rate sensitive to measurement errors since only a few DO data points are available.

Potential difficulties with this technique are that during the DO decline, oxygen transfer from the gas phase to the liquid phase needs to be avoided (especially critical when the respiration rate is low) and that identifying a linear decrease of DO is not always evident. The latter is especially a challenge when the measuring technique is automated. In fact, the transition from the reaeration phase to the DO decline phase can take some time (tens of seconds) and is affected by the removal of gas bubbles from the liquid and by the transient response of the DO probe. Respirometers based on this technique allow the measurement of the respiration rate with a measuring interval ranging from typically a few minutes to several tens of minutes. They also permit, especially in the lab, the generation of respirograms by the addition of wastewater or specific substrates. Figure 3.18 is an illustration of a respirometer based on this technique. This floating ball respirometer is designed for automated sampling and discharge of activated sludge, repeated aeration and calculation of respiration rate.

Figure 3.17 Raw DO concentration data from a LSS respirometer with reaeration.

Figure 3.18 Example of a practical implementation of respirometry following the LSS principle. (A) a close up with a DO probe visible and (B) the respirometer in place, i.e. floating on the activated sludge in an aeration tank (photos: Strathkelvin Instruments Ltd.).
Figure 3.19 Example of another practical implementation following the LSS principle (photo: P.A. Vanrolleghem).

Figure 3.19 is an example of another practical implementation following the LSS principle with a closed respiration cell (the right-hand vessel) that is filled with activated sludge from the aerated tank (the left-hand vessel). The DO decline in this cell is measured until a certain minimal DO is reached (or after a given time, or a given DO variation), after which the content is exchanged for fresh, aerated activated sludge from the aerated vessel and a new cycle is started.

- Liquid phase, flowing gas, static liquid (LFS) principle

The disadvantage of the need for reaeration can be eliminated by continuously aerating the biomass. The continuous supply of oxygen guarantees a non-limiting DO concentration even at high respiration rates, for example at high biomass concentration and high wastewater or substrate doses. Moreover, continuous aeration allows an open vessel, which facilitates the addition of wastewater and substrate.

To obtain the respiration rate, both the mass transfer term (under process conditions) and differential term in the DO mass balance must be known (Eq. 3.3). The mass transfer term is calculated from the measured DO concentration, the mass transfer coefficient kLa and the DO saturation concentration $S_{O_2}^*$. These two coefficients have to be determined regularly because they depend on environmental conditions such as temperature, barometric pressure and the properties of the liquid (e.g., salts and certain organics). The simplest approach is to determine these by using separate reaeration tests and look-up tables. Standard procedures for these tests under process conditions are available and are based on the disturbance of the equilibrium DO concentration by interrupting the aeration, adding hydrogen peroxide, or even by the addition of a readily biodegradable substrate. The obtained reaeration curve can then be used to estimate the kLa and DO saturation concentration $S_{O_2}^*$. Nonlinear parameter estimation techniques as presented in Chapter 5 are recommended to obtain reliable values.

The advantage of the method based on disturbance by respiration of a readily biodegradable substrate is that the values of the aeration coefficients can be updated relatively easily and frequently. However, when only a moderate DO disturbance occurs, the accuracy of the estimated kLa is low. Also, it must be assumed that the respiration rate has dropped to a constant (endogenous) rate during the reaeration part of the curve.

Estimation of $S_{O_2}^*$ is not required when one is only interested in the substrate-induced respiration, i.e. exogenous respiration $r_{O_2,exo}$. Total respiration is the sum of endogenous respiration $r_{O_2,endo}$ and exogenous respiration $r_{O_2,exo}$. Considering $r_{O_2,endo}$, kLa and $S_{O_2}^*$ to be constant over a short interval, it can be shown that the equilibrium DO concentration reached under endogenous conditions $S_{O_2,endo}^*$ encapsulates the endogenous respiration (Kong et al., 1996). The mass balance for oxygen can then be rewritten as:

$$\frac{dS_{O_2}}{dt} = kLa \cdot (S_{O_2}^* - S_{O_2}) - r_{O_2} = kLa \cdot (S_{O_2,endo}^* - S_{O_2}) - r_{O_2,endo} - r_{O_2,exo} \quad \text{Eq. 3.13}$$

By putting $r_{O_2,endo} = kLa (S_{O_2,endo}^* - S_{O_2})$ and replacing $r_{O_2,endo}$ in the above equation, one obtains:

$$\frac{dS_{O_2}}{dt} = kLa \cdot (S_{O_2,endo}^* - S_{O_2}) - r_{O_2,exo} \quad \text{Eq. 3.14}$$

To estimate the exogenous respiration rate $r_{O_2,exo}$ from this mass balance, one therefore only needs to estimate the equilibrium DO concentration $S_{O_2,endo}^*$ (directly from the data, Figure 3.20) and the kLa from the reaeration part of a DO disturbance curve obtained with a readily biodegradable substrate. Another advantage of this LFS respirometric principle is that it allows the measurement of $r_{O_2}$ at a nearly constant DO concentration, thereby eliminating the dependency of the respiration on the DO concentration (provided $DO >> 0 \text{ mg L}^{-1}$). Yet another advantage is that the time interval at which respiration rate values can be obtained is short, and is in fact only limited by the measuring frequency of the DO probe. This makes a respirometer based on the LFS principle suitable for kinetic tests and model optimization experiments.
RESPIROMETRY

Figure 3.20 (A) Diagram showing a LFS principle-based respirometer setup (Vanrolleghem et al., 1994) and (B) an example of typical raw data (Kong et al., 1996).

Obviously, whereas in its basic form a respirometer following the LFS principle consists of a vessel equipped with an aerator, an impeller or recirculation pump for mixing and a DO probe, a more advanced version designed for automated (online) experiments includes supplementary equipment such as pumps for filling the vessel with biomass, wastewater and substrate addition, level switches, and valves for vessel drainage. This requires a suitable data handling and control system, besides sufficient computing capacity for the estimation procedure. Figure 3.21 shows an example of a commercial version of this measuring principle.

Figure 3.21 Example of a commercial version of a LFS principle-based respirometer using the setup depicted in Figure 3.20. In the middle is the thermostated vessel and at the bottom, left and right, pumps for wastewater and calibration addition (photo: Kelma NV).

- **Liquid phase, static gas, flowing liquid (LSF) principle**

The LSF respirometric measuring principles allow continuous sampling of a biomass stream, for example activated sludge from a lab reactor or full-scale aeration tank, while measuring the respiration rate. When the biomass flows through a closed completely mixed vessel (respirometric cell) without a gas phase, then, following Eq. 3.4, the respiration rate can be calculated if the volume of the respirometric cell and the flow through rate are known and DO concentrations at the inlet and outlet of the cell are measured. Alternatively, the DO can be measured in the source reactor (provided that the
decrease in DO in the supply line is negligible) and in the
respirometric cell itself. In any case, as with other
principles the DO concentration in the cell must be high
enough to prevent DO limitation, which requires a
sufficiently high input DO concentration at a given
respiration rate. A potential source of erroneous
measurements is associated with the use of two DO
probes to measure DO at the inlet and outlet, because
when probe characteristics differ slightly, relatively large
relative errors can occur in the difference between the
two DO measurements needed to calculate the respiration
rate. Spanjers and Olsson (1992) solved this by
measuring the DO concentrations in the inlet and outlet
of the cell alternately with one single probe located at one
port. This was realized by periodically changing the flow
direction through the vessel using four solenoid valves
which were activated two by two (Figure 3.22).

If a steady state is assumed with respect to the
respiration rate then the rate can be calculated using Eq.
3.4 by assuming that the derivative is zero. However, by
approximating the equation by a difference equation, the
respiration rate can be calculated under dynamic
conditions.

Figure 3.23 shows a commercial version of the LSF
principle using the one probe solution. This respirometer
measures the respiration rate with an interval of typically
one minute and can be connected to a lab reactor or a full-
scale aeration tank, for example by using a fast loop.

- The gas phase, static gas, static liquid (GSS) principle

Like the LSS principle which is one of the liquid phase
principles, the GSS principle is the simplest gas phase
principle because the absence of flowing gas and liquid
implies that no supplementary materials, such as pumps
and aeration equipment, are needed. However, because
the calculation of the respiration rate is based on the
measurement of oxygen in the gas phase and the actual
respiration takes place in the liquid phase, the relation
between the gas phase oxygen dynamics must be related
to the respiration rate. Thus, in addition to the DO mass
balance in the liquid phase, an oxygen mass balance in
the gas phase must be considered and the set of equations
solved for the respiration rate, assuming a transfer
relation between the gas and liquid.

Figure 3.22 Example of a practical implementation following the LSF
principle, with one DO probe to measure DO concentration at the inlet and
outlet of the respiration vessel (Spanjers, 1993). (A) A schematic of the
measurement arrangement and (B) A typical profile of a signal recorded by
the single probe (Spanjers and Olsson, 1992). The signal represents the DO
oscillating between the DO at the inlet and the DO at the outlet of the cell.
This DO signal is the basis for the calculation of the respiration rate.

Figure 3.23 Example of a commercial version of the LSF principle using the
setup depicted in Figure 3.22. On the left is a lab-scale aeration tank with
activated sludge. In the equipment box on the left is the sampling pump,
and on the right is the set of solenoid valves. The vessel is placed behind
the valves (photo: Applitek NV).
A typical GSS-based respirometer consists of a biomass vessel, mixing equipment and a gas measurement arrangement. Gaseous oxygen is measured by physical methods, such as the gasometric method or the paramagnetic method. Gasometric methods measure changes in the concentration of gaseous oxygen, which can be derived from changes in the pressure (if volume is kept constant, a manometric method) or changes in the volume (if pressure is kept constant, a volumetric method, e.g. Mariotte’s bottle), see Section 3.3.1.2. As with the LSS principle, when the oxygen consumption is too high, these methods need replenishment of the gaseous oxygen and thus temporary interruption of the measurements. This limits the possibility for continued monitoring of the respiration rate. An important complication of the GSS principle is that, because carbon dioxide is released from the liquid phase as a result of the biological activity, this gas has to be removed from the gas phase in order to avoid interference with some of the simpler oxygen measurement principles. In practice this is done by using alkali to chemically absorb the carbon dioxide produced.

Similarly to the LSS principle, the GSS principle can be used to carry out a BOD test (Section 3.4.2), for example Oxitop (Figure 3.16).

- The gas phase, flowing gas, static liquid (GFS) principle
Like the LFS measuring principle, respirometers using the GFS principle are based on a flowing gas phase, i.e. the biomass is continuously aerated with air (or pure oxygen) so that the presence of sufficient oxygen in the liquid is ensured. However, the calculation of the respiration rate is based on measurement of oxygen in the gas phase, more specifically the gas leaving the liquid phase after aeration, also called off-gas. Because the off-gas may contain other components that are influenced by the metabolic processes in the liquid phase, such as carbon dioxide and nitrogen, it is obvious that these may also be measured to obtain additional information on the activity of the biomass.

A typical GFS-based respirometer consists of a biomass vessel, aeration and mixing equipment and an off-gas measurement arrangement. Gaseous oxygen is measured by physical methods, such as the gasometric method (that is: by supplying pure oxygen from a reservoir or by using electrolysis) or the paramagnetic method.

Following the gasometric method, in a closed headspace the change of pressure or change of volume is related to oxygen consumption in the liquid phase. This information can be used to activate an oxygen production system, based on an oxygen bottle or an electrolytic cell, and the oxygen flow or electrical current can be converted to the respiration rate. In fact the resulting oxygen supply serves as the aeration, i.e. flowing gas. Change of pressure can be measured by a pressure sensor. No documentation exists that describes the use of volume change to activate oxygen supply. Obviously the gasometric method is based on measurement of the oxygen transfer \([k_L \cdot (S_O^* - S_O)]\) and not on the measurement of the oxygen concentration in the gas phase. The gas phase oxygen concentration must be assumed constant (i.e. \(dC_O / dt = 0\)). In any case the gasometric method requires a \(CO_2\) absorption arrangement in order to remove \(CO_2\) from the gas. \(CO_2\) in the gas that is produced during biodegradation and is released to the gas phase from the liquid phase, would otherwise interfere with the measurement of pressure or volume change. Typically one mole of \(CO_2\) is produced per mole of \(O_2\) in aerobic respiration, which by definition means that no gas pressure change will occur.

Alternatively, the oxygen concentration in the gas phase may be measured directly, which eliminates the need to capture \(CO_2\) and allows continuous aeration of the biomass with air. However, in addition to the gaseous oxygen concentration, \(DO\) concentration in the liquid phase must be measured because besides the mass balance in the gas phase, the mass balance in the liquid phase also needs to be considered. Likewise, the flow rate of the gas needs to be measured, for example with a mass flow controller.

Oxygen is one of the few gases that show paramagnetic characteristics, so it can be measured quantitatively in a gaseous mixture by using the paramagnetic method. The method is based on the change in a magnetic field as a result of the presence of oxygen, and this change is proportional to the concentration of gaseous oxygen.

Another method to measure oxygen concentration in the gas phase is by using a mass spectrometer. Using this more expensive equipment has the advantage that other gases may also be measured, which then is usually termed off-gas analysis. Especially \(CO_2\) may be measured in the gas phase because it is a useful indicator of biomass activity under all the redox conditions. However, if \(CO_2\) in the off-gas is measured, and because \(CO_2\) is associated with the carbonate system, extra equipment will be needed to measure the pH and bicarbonate concentration in the liquid phase (Pratt et al., 2003). Note that a mass spectrometer is expensive.
equipment that also requires special (calibration) gases, which makes this method more an advanced laboratory tool rather than a field application. An alternative may be a less expensive infrared CO₂ analyser.

Figure 3.24 shows an example of a practical implementation of the GFS principle. This respirometer is based on off-gas analysis using mass spectrometry and is integrated with a titration unit to account for the interaction of CO₂ production and evolution with the acid/base buffering systems in the liquid phase.

The respirometer was used to examine the two-step nitrification process, more specifically nitrite accumulation in wastewater treatment systems operated under varying environmental conditions, i.e. pH and DO concentrations (Gapes et al., 2003).

3.4 WASTEWATER CHARACTERIZATION

Several methods have been developed and applied for the characterization of wastewater, both in terms of pollutant load characterization and in terms of toxicity assessment. In what follows first the different respirometric methods for evaluation of biochemical oxygen demand will be described, followed by respirometric toxicity tests and, finally, an overview of the methods for wastewater fractionation.

3.4.1 Biomethane potential (BMP)

3.4.1.1 Purpose

A biomethane potential (BMP) test is performed when the methane yield of a substrate needs to be known, e.g. when a business case for an anaerobic digester is being developed. Also, the methane production over time can be of interest to optimize the solids retention time of the digester or for the dimensioning of the biogas handling equipment.

3.4.1.2 General

The BMP test is performed to test what the methane potential of a sample is. BMP determines, to a certain extent, both the design and economic details of a biogas plant (Angelidaki et al., 2009). But the BMP test can also be done to evaluate the performance of the biomass. For example, the methane production rate can be used to estimate the hydrolytic activity of the inoculum. BMP tests are often used in literature but there is a large variety in protocols. There have been some efforts to propose a standard (Angelidaki et al., 2009).
Figure 3.25 depicts a typical result of a BMP test. In this case the methane production over time of coarsely filtered sewage is displayed.

Figure 3.25 Results of the BMP test: methane production over time from coarsely-filtered sewage. Measurements were carried out in triplicate. The red line represents solids from the filter belt; green, solids from the drum filter, and blue, a mix of both solids (Kooijman, 2015; unpublished data)

### 3.4.1.3 Test execution

In BMP tests, the amount of inoculum (required for anaerobic digestion) is usually measured as volatile suspended solids (VSS). Volatile solids (VS) is a possible alternative. The difference between these methods is that VS considers all the volatile fractions in the inoculum, whereas VSS only considers solids larger than a certain mesh, separated by filtration. VSS is preferred over VS because viable biomass is not expected to pass through the filter in the VSS measurement and thus VSS is more closely correlated to viable biomass than VS; see also Section 3.5.1. However VS measurement can also be chosen because of its accuracy and simplicity of measurement and generally low dissolved volatile solids concentration in inoculum, compared with the volatile particulates. For VS or VSS measurement, most researchers use the standard method (APHA et al., 2012).

In BMP tests, the way to measure the substrate depends on the purpose of the test and on the form of the substrate. For wastewater biomass usually VS is used. Liquid substrate such as wastewater can be quantified by COD. When the BMP in conventional anaerobic digestion is investigated, it is important that no VFAs are accumulated during the BMP tests. This means that the acidification and VFA consumption by methanogenic consortia need to be in equilibrium. The ratio between inoculum and substrate is therefore important. For biomass from a digester the following ratio can be applied, which will in most cases not lead to a net VFA production:

$$2 \geq \frac{VSS_{inoculum}}{VS_{substrate}}$$

Eq. 3.15

Notice that these are masses applied in the test, expressed in grams of VS or VSS, not concentrations. To obtain the BMP of the substrate, the background production of methane from the inoculum (a blank without substrate) needs to be measured in parallel and subtracted from the produced gas of the inoculum-substrate mixtures.

To perform the BMP test, the reaction bottles need to be incubated at the desired temperature. The standard of mesophilic temperature is 35 °C. The temperature can be kept constant by using a water bath. Stirring has to be performed by impellers in the reaction bottle. When using biomass with high viscosity in BMP tests, insufficient stirring may negatively affect the digestion rates and sometimes impellers in small test bottles (200-400 mL) do not meet the stirring requirement. A solution for this can be to incubate the reaction bottles in an incubator shaker. The duration of the BMP tests depends on their purpose and the characteristics of the substrate but it is most common to use a run time of 30 days.

### 3.4.1.4 Data processing

In many cases anaerobic respirometric tests aim at measuring the BMP of a substrate. As shown in Figure 3.25, the BMP depends on the time of digestion, similar to the analysis of BOD (Section 3.4.2). The BMP is also usually expressed per gram of VS. For waste-activated sludge this is between 150-200 N-mL gVS⁻¹. For primary sludge this is 300-400 N-mL g VS⁻¹. Since the hydrolysis is often the rate-limiting step in anaerobic digestion (Eastman and Ferguson, 1981), the methane production rate is directly related to the hydrolysis rate and thus the slope of a respirogram as shown in Figure 3.25 is a direct measure of the hydrolysis rate at that point in time.

### 3.4.1.5 Recommendations

- **Pressure and temperature correction**

In order to determine the amount of methane that is produced in an experiment, the volume in combination
with the pressure and temperature is required at each time instant during a test. Typically the amount of produced gas is expressed under standard conditions (usually 273.15 K, 0 °C and 1,013.25 mbar, 1 atm).

- **Methane diffusion**
  Methane molecules are known to be able to diffuse through plastics such as silicon. Therefore it is crucial in the design of a BMP (or Specific Methanogenic Activity, SMA, Section 3.5.2) test that the materials that are in contact with the biogas, have poor diffusivity for methane.

- **pH indicator dye for the scrubbing solution**
  It is crucial that the liquid in the scrubber bottles and in the measurement device as depicted in Figure 3.13 and Figure 3.15, respectively, has a high (> 9) pH such that CO₂ and H₂S are absorbed by the liquid. A lower pH will result in erroneous measurements. To ensure that the scrubbing liquid is not saturated, methylene blue dye can be added, which turns the liquid blue when pH > 9 making it possible to visually verify the effectiveness of the scrubber solution.

- **Inoculum activity**
  The activity of methanogens is very prone to temperature differences. Especially when performing SMA tests, it is important that the activity of the methanogens is high from the beginning of the experiments. Therefore it is strongly advised to store the inoculum at 35 °C for 24 h prior to the experiment. In this way, in a BMP (or SMA) test performed at 35 °C, there will be no temperature shock for the methanogens and the BMP (or SMA) will not be affected by temperature.

- **Micro and macro nutrients**
  During a BMP (or SMA) test there may be a lack of micro and macro nutrients, which may affect the conversion performance. If insufficient nutrients are present they should be added. In literature there are various suggestions for nutrient solutions for anaerobic digestion tests (Angelidaki et al., 2009; Zhang et al., 2014).

- **Oxygen inhibition**
  When water is in equilibrium with air, the concentration of oxygen will be around 9 mg L⁻¹ at room temperature and sea level. Oxygen is known to inhibit methanogens. Also oxygen will ‘consume’ COD in a mixture. Therefore, it can be desirable (especially in SMA tests where the rates of methanogens are measured) to remove the oxygen from the substrate solution before mixing with biomass. Flushing with N₂ gas is commonly applied. Very gently bubbling N₂ gas for ~ 60 sec through the substrate solution will remove oxygen.

- **Gas tightness**
  Prior to the BMP test, the system of gas production and gas measurement should be verified to be gas tight. This can be done by injecting a known quantity of air into the tube connected to the scrubber and gas flow meter to ensure that everything is mounted correctly and gas tight. The amount of air measured and the amount of air injected must be equal. If not, there is probably a leak.

- **Alkaline scrubbers**
  Alkaline scrubbers are typically used to remove acid components from gas. Other compounds such as NH₃ and H₂ are not removed in alkaline scrubbers. NH₃ is often present in significant amounts when the pH of the digestate is high (close to 9). When there is a severe acidification in the digester, H₂ may be formed and thus be present in larger quantities in the biogas. To avoid substantial variations in the pH of the mixture during the assay, a phosphorus buffer solution may be added to the mixture.

### 3.4.2 Biochemical oxygen demand (BOD)

#### 3.4.2.1 Purpose

The biochemical oxygen demand (BOD) test is performed to assess the biodegradable organic matter concentration of a water sample, e.g. to design a wastewater treatment plant (WWTP) or to evaluate its performance in terms of organic matter removal. Thanks to its sensitivity, it is also used to evaluate the organic matter concentration of the receiving waters.

#### 3.4.2.2 General

The determination of the BOD of wastewaters, effluents, and polluted receiving waters is based on a test that measures the bacterial consumption of oxygen during a specified incubation time. The test quantifies the biochemical degradation of organic material (carbonaceous biochemical oxygen demand - CBOD) but it will also include the oxygen used to oxidize inorganic material such as sulphides and ferrous iron. Unless a nitrification inhibitor is added in the test, it may also measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous biochemical oxygen demand - NBOD). To make clear what is meant, the term ‘total BOD’ (BODₜ) is used when no nitrification inhibitor is added, i.e. the sum of CBOD and NBOD.
Normally the incubation time is limited to five days, leading to the traditional BOD. However, tests can be conducted over other incubation times, e.g. seven days to facilitate lab organization, or 28, 60 up to 90 days of incubation to determine the so-called ultimate BOD (also: UBOD, BOD∞ or BODU). This measures the oxygen required for the total degradation of organic material (the ultimate carbonaceous demand) and/or the oxygen to oxidize reduced nitrogen compounds (the ultimate nitrogenous demand).

Measurements that include NBOD are not generally useful for assessing the oxygen demand associated with organic material. In fact, NBOD can be estimated directly from nitrifiable nitrogen (ammonia or total Kjeldahl nitrogen) and CBOD can be estimated by subtracting the theoretical equivalent of the reduced nitrogen oxidation from the uninhibited test results. However, this method is cumbersome and is subject to considerable error. The chemical inhibition of nitrification provides a more direct and more reliable measure of CBOD.

In other methods oxygen is supplied continuously from a gas phase present in the bottle (LFS respirometer) and the oxygen consumed is monitored.

In what follows the first test approach will be presented and subsequently the methods using oxygen supply from a gas phase are discussed.

- **BOD test with a LSS respirometer**
  The method consists of completely filling an airtight bottle with a water sample and incubating it in the dark (to prevent photosynthesis) at 20 ± 0.1 °C for a specified number of days (5, 7, 28, 60, 90 days). Dissolved oxygen concentration is measured initially and after incubation, and the BOD is computed from the difference between the initial and final DO. Because the initial DO concentration is determined shortly after the dilution is made, all the oxygen uptake occurring after this measurement is included in the BOD measurement. The bottles are typically 300 mL with a ground-glass stopper and a flared mouth. Bottles should be cleaned carefully with detergent and rinsed thoroughly (to eliminate any detergent from the bottle).

**Dilution**
Since the only source of oxygen is that initially present, only limited oxidation can take place because DO concentration should never decrease below 2 mg O2 L⁻¹ to prevent oxygen limitation. This therefore limits sample BOD concentrations to about maximum 7 mg L⁻¹. Whereas this may be adequate for effluent and receiving water samples, wastewaters will have to be considerably diluted in this closed bottle test. Dilution comes however with problems since bacterial growth requires nutrients such as nitrogen, phosphorus, and trace metals (Mg, Ca, Fe). Without these the biodegradation of the pollutants may be limited, leading to underestimation of the BOD. Also, buffering may be needed to ensure that the pH of the incubated sample remains in a range suitable for bacterial growth. Obviously the dilution water used should not contain biodegradable matter.

**Seeding**
Since the test depends on bacterial activity to degrade the organic matter present in the sample, it is essential that a population of microorganisms is present that is capable of oxidizing the biodegradable matter in the sample. Domestic wastewater, non-disinfected effluents from biological WWTP and surface waters subjected to wastewater discharge contain satisfactory microbial populations. Some waters, e.g. industrial wastewaters, may require a ‘seed’ to initiate biodegradation. Such a seed can be obtained from the biomass or effluent of a...
WWTP, but since nitrifiers may be present in such seed, it is recommended to apply a nitrification inhibitor in the test to ensure proper CBOD test results. In some cases the pollutants may require organisms other than the ones present in domestic WWTP and then it is recommended to seed with bacteria obtained from a plant that is subject to this waste or from receiving water downstream the discharge point.

Blank

Both the dilution water and seed may affect the result of the BOD test, e.g. by introducing organic matter into the bottle. In fact four situations may occur as illustrated in the scheme below:

<table>
<thead>
<tr>
<th></th>
<th>Seed</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>x</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>4</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

Since the test quality could be affected by dilution, it should be ensured by performing a blank BOD test in which the same amount of seed as in the sample test is added to a bottle filled with water for dilution (Figure 3.27).

DO measurement

Dissolved oxygen can be measured with the azide modification of the titrimetric iodometric method or by using a well-calibrated DO electrode. For the measurement of the ultimate BOD over extended incubation periods, only the DO electrode measurement approach is recommended because DO must be measured intermittently during the incubation (intervals of 2 to maximum 5 days, minimum 6 to 8 values).

Data processing

The calculation of the BOD of the sample is as follows (Figure 3.27):

$$BOD = \frac{(D_1 - D_2) - (B_1 - B_2)}{P}$$  \hspace{1cm} Eq. 3.16

Where, $D_1$ is the DO concentration of the diluted sample immediately after preparation (mg L$^{-1}$), $D_2$ is the DO concentration of the diluted sample at the end of the incubation period (mg L$^{-1}$), $B_1$ is the DO concentration of the blank immediately after preparation (mg L$^{-1}$), $B_2$ is the DO concentration of the blank at the end of the incubation period (mg L$^{-1}$), and $P$ is the decimal volumetric fraction of the sample used.

Note that in fact the mass balance of the closed bottle test is written as (the LSS respirometric principle):

$$\frac{dS_{O_2}}{dt} = -r_{O_2}$$  \hspace{1cm} Eq. 3.17

From which follows that, after integration,

$$BOD = S_{O_2,t_0} - S_{O_2,t_{fin}} = \int_{t_0}^{t_{fin}} r_{O_2}(t) \cdot dt$$  \hspace{1cm} Eq. 3.18

Showing that the BOD is nothing else but the area under a so-called respirogram, i.e. a time series of respiration rate data.

For the determination of the ultimate BOD, the following first order equation should be adjusted to the time series of DO depletion data (Figure 3.28):

$$BOD_t = BOD_u (1 - e^{-kt})$$  \hspace{1cm} Eq. 3.19

Where, $BOD_t$ is the oxygen uptake measured at time $t$ (mg L$^{-1}$), $BOD_u$ is the ultimate BOD (mg L$^{-1}$), and $k$ is the first order oxygen uptake rate coefficient (d$^{-1}$).
Equation adjustment should preferably be performed using nonlinear regression, e.g. by using the Solver function in Excel to minimize the sum of squared errors between the measured time series of oxygen uptake and the model predictions of BOD\(_t\) for certain BOD\(_U\) and k values. Note that this approach not only yields BOD\(_U\) but also k. The latter provides information on the degradation rate of the organic matter. Note that a first-order model may not always be the best choice. Much better fits can often be obtained with alternative kinetic models, in particular consisting of a sum of two or more first-order models.

**Recommendations**

When the DO concentration at the end of the test is below 1 mg L\(^{-1}\) or the DO depletion is less than 2 mg L\(^{-1}\), the test should be carried out again, but with a higher or lower dilution, respectively.

To verify whether the BOD test has been performed well, a test check can be made with a solution with known BOD. The recommended solution is a standard mixture of 150 mg L\(^{-1}\) glutamic acid and 150 mg L\(^{-1}\) glucose. A 2% dilution of this stock solution should lead to a BOD\(_U\) of approximately 200 ± 30 mg L\(^{-1}\). A BOD\(_U\) of 308 mg L\(^{-1}\) can be anticipated (APHA, 2012).

Sample pre-treatment may be necessary. The temperature and pH of the sample may have to be adjusted to 20 °C and 6.5 < pH < 7.5 before dilution. If the sample has been chlorinated, it should be dechlorinated (by adding Na\(_2\)SO\(_3\)) and a seed should certainly be used. Samples supersaturated with oxygen (above 9 mg L\(^{-1}\) at 20 °C) may be encountered when the sample was cold or there has been photosynthetic activity. In such cases deoxygenation is necessary by vigorously shaking a partially filled BOD bottle or by aerating it with clean compressed air.

Nitrification can be inhibited using multiple chemicals, e.g. nitrapyrin, allylthiourea (ATU), or 2-chloro-6-(trichloromethyl)pyridine (TCMP). While recommended concentrations normally lead to adequate inhibition, adaptation to these chemicals has been reported and they can also be degraded during the test, allowing nitrification to start up at a later stage in the test. It is therefore recommended to check at the end of the test whether nitrite and nitrate have been formed.

(Field) test kits are available to measure BOD without lab equipment. Standard nutrients and seed bacteria are supplied, and DO is measured based on the photometric method.

- **A BOD test with a GFS respirometer**

To solve the problem of a limited amount of dissolved oxygen available in the closed bottle test, BOD test equipment has been developed. The oxygen is supplied to the liquid within this test equipment, thus enabling the degradation of organic matter. In this way, dilution may not be necessary or reduced significantly as the BOD measurement range can be extended significantly. The basic equipment consists of a bottle in which a gas phase is provided that can supply oxygen as it is consumed in the tested sample. Either the volume of gas contains sufficient oxygen to allow completion of the oxidation of the biodegradable matter in the sample, or the equipment is able to replenish the gas phase with fresh oxygen from an external source. In this way, besides BOD, the oxygen uptake can also be measured more or less continuously over time, which inherently provides the possibility to calculate the respiration rate over time.

There is various equipment that works according to the GFS principle. Basic principles include manometric respirometers that monitor the pressure change in the gas phase above the liquid as oxygen is consumed. Interference by CO\(_2\) that is produced during biodegradation and released from the liquid is dealt with by capturing the CO\(_2\) in an alkaline (KOH) solution or granules integrated in the equipment. Using the ideal gas law the recorded pressure drop can be translated into oxygen uptake. Equipment using a pressure transducer, simple calculation logic and a data logger for the BOD time series is commercially available. Volumetric respirometers record the gas volume reduction (at constant pressure) as oxygen gets depleted in the gas phase. Again, CO\(_2\) scrubbing with alkaline solution/granules is required. Electrolytic respirometers
use the principle of constant volume and gas pressure to activate the electrolytic production of oxygen and maintain the oxygen concentration in the gas phase constant (Figure 3.29). CO₂ emitted from the liquid would again interfere with the constant pressure principle and must be eliminated from the gas phase. Alternatively, oxygen may be supplied, and its flow be measured, from a pure oxygen source (e.g. gas bottle) to maintain pressure in the airtight bottle.

![Figure 3.29 An electrolytic respirometer for BOD analysis (SELUTEC GmbH).](image)

If no external oxygen supply is available, as in the respirometer described earlier (“BOD test with a LSS respirometer”), one should pay attention to the overall oxygen consumption that will be exerted by the sample. This should not exceed the amount of oxygen present in the gas phase above the liquid as this would lead to oxygen limitation and thus erroneous results. The volume of sample to be added in the bottle will thus depend on its BOD content and respirometer manuals will typically provide a table with volumes to be added for different BOD ranges. In any case, methods without oxygen supply suffer from decreasing DO concentration in the liquid, which may influence the oxidation rate during the test.

The instruments allow readings of the BOD as frequent as every 15 min to every 6 h. High frequency data collection may help in interpreting the results in terms of degradation kinetics or allow a better model to fit to reduce the influence of measurement noise or get a more reliable estimate of the ultimate BOD.

**Recommendations**

Interference by gases other than CO₂ may lead to erroneous results, but is not often reported. Temperature variations may also affect pressure and volume measurement as they affect overall pressure. In addition, atmospheric pressure changes may affect the measurement in some respirometers.

An oxygen demand as small as 0.1 mg L⁻¹ can be detected, but the test precision will depend on the total amount of oxygen consumed, the precision of the pressure or volume measurement and the effect of temperature and atmospheric pressure variations.

Since oxygen must be transferred from the gas phase to the liquid phase, one must be careful that the oxygen
uptake rate does not exceed the mass transfer rate by too much as this would lead to oxygen limitation of the biodegradation process and thus errors in the BOD result. The oxygen transfer is mostly dependent on the mixing conditions in these respirometers, thus limiting the oxygen uptake rates to 10 mg L⁻¹ h⁻¹ for low mixing devices and to 100 mg L⁻¹ h⁻¹ if high intensity mixing is provided. If the uptake rate exceeds the supply rate of the respirometer at hand, one can dilute the sample such that the uptake rate decreases to acceptable values. The dilution water composition must be checked as mentioned above.

Note that the BOD test is a respirometric test because it measures consumption of the terminal electron acceptor O₂, although it generally does not measure the respiration rate. In fact BOD is the cumulative oxygen consumption that may be obtained by integrating the respiration rate over a certain incubation period. As noted above, the LFS respirometry inherently provides the possibility to calculate the respiration rate over time.

Also note that BOD can be measured in similar ways by using other electron acceptors, such as nitrate.

Seeding and nutrient additions may be required for particular wastewater samples if competent biomass is not present or the wastewater is not balanced in terms of nutrients versus organic material and may thus be subject to limitations of bacterial growth and thus biased BOD results.

### 3.4.3 Short-term biochemical oxygen demand (BODₜₜ)

Temporal variation of the wastewater composition can be readily characterized by using chemical methods such as COD and TOC analysis. These methods can provide data at high measuring frequency (e.g. on an hourly basis) but they do not provide information on the bio-treatability of the pollutants. Traditional methods that rely on the monitoring of the biodegradation of the pollutants to obtain an indication of the treatability such as the aforementioned BOD₅ method are clearly unsuitable to provide such high-frequency information due to the large time delay between the sample introduction and measurement result. However, the principle of monitoring the oxygen uptake for assessment of the treatability and organic matter concentration of a wastewater is a very powerful one, because most wastewater treatment processes rely on aerobic degradation of the organic matter. Therefore, methods have been proposed to decrease the response time of these biologically mediated methods to such a level that their application in high-frequency monitoring becomes possible.

The short-term biochemical oxygen demand (BODₜₜ) is defined as the amount of oxygen consumed for biodegradation of readily biodegradable organic matter per volume of wastewater.

In the traditional BOD₅ test (Section 3.4.2), a small amount of biomass is added to a large wastewater sample (typically the initial substrate to biomass ratio S₀/X₀ is set to be between 10/1 and 100/1 mg BOD₅ mg MLVSS⁻¹). As a result, substantial growth has to occur before the available pollutants are degraded and possibly a lag phase may occur where adaptation of the sludge to the pollutants takes place. To speed up the response time (to within an hour), the techniques for BODₜₜ determination are based on a low S₀/X₀ ratio (typically 1/20 to 1/200 mg BOD₅ mg MLVSS⁻¹). These conditions are obtained by the addition of a small aliquot of wastewater to the activated sludge present in the test vessel. In this way the degradation time can be reduced considerably (to often less than one hour) and no significant growth of biomass is to be expected given the relatively low amount of organic matter added.

Because of this short test time, it is evident that matter that biodegrades slowly in the wastewater sample will not be degraded, i.e. only the readily biodegradable fraction is measured. There is also no time for adaptation of the biomass to any new organic component that may be present in the wastewater and to which the biomass is exposed for the first time. In addition there is an important issue of not using biomass from an enhanced biological sludge removal (EBPR) plant. This biomass contains phosphorus accumulating organisms (PAO) that can store volatile fatty acids (VFA). When the VFA fraction of the readily biodegradable matter is taken up for storage it will not be oxidized and hence it will not be measured in a respirometric test. The reader is also referred to sections 3.5.3.2 and 3.5.3.3 (Figure 3.48).

However, being able to measure the readily biodegradable matter is very relevant information in particular for anticipating and optimizing the performance of denitrification and enhanced biological phosphorus removal. Note that, because a respirometric test can be used to distinguish between slowly and readily biodegradable matter, this provides a basis for the assessment of wastewater fractions in the context of a mathematical model (Section 3.4.5).
Due to the increased respiration resulting from the high degradative capacity available in the test vessel, problems may arise to meet the oxygen requirements of the sludge. Respirometric methods that are based on measurement of the decrease of the initial amount of oxygen present in the biomass are severely restricted because of the risk of oxygen limitation. As a result the dynamic concentration range of such methods is small, with maximum sample additions of 5 mg BOD₅ L⁻¹ activated sludge. Obviously, aeration of the sludge, available in many respirometric equipment, solves this problem. However, the intensity of aeration must be sufficient. Still, since in many cases the dissolved oxygen is measured in the test vessel, it can easily be checked whether the oxygen supply has been sufficient (that is: DO should always be above 2 mg L⁻¹) during the BOD₅ test. Initial BOD₅ concentrations in the test vessel can easily reach 100 mg BOD₅ L⁻¹ in most respirometers.

### 3.4.3.1 Test execution

The BOD₅ test is performed by first bringing a volume of activated sludge in the thermostated (e.g. 20 °C) and aerated test vessel and letting it come to a stable state characterized by what is called endogenous respiration. Endogenous respiration is defined as the state the biomass gets in when there is no more external substrate available in the activated sludge. The biomass is respiring its own reserve materials or is respiring the lysis products of dead biomass. Reaching this state may take between a few hours and a day, depending on how loaded the activated sludge is with slowly biodegradable (hydrolysable) matter. Typical endogenous respiration rates are between 2 and 10 mg O₂ L⁻¹ h⁻¹. Once the endogenous state has been reached a pulse of wastewater is injected, the amount of which is calculated to lead to the desired S₀/X₀-ratio of approximately 1/20 to 1/200 mg BOD₅ mg MLVSS⁻¹. As soon as the substrate becomes available to the biomass, biodegradation starts and the oxygen uptake rate increases quickly to a maximum rate that is determined by the activity of the biomass and the degradation rate of the substrate. If nitrifiers are present in the sludge and the wastewater contains nitrifiable nitrogen (ammonia and organic nitrogen that is ammonified rapidly) this exogenous respiration rate also includes the oxygen uptake rate for nitrification. The substrates will gradually get exhausted and the oxygen uptake rate will gradually decrease to eventually reach the endogenous rate. The substrate pulse-induced curve of oxygen uptake rates is called a respirogram, a schematic of which is given in Figure 3.30.

![Figure 3.30 Schematic of a respirogram. After biomass has undergone endogenous respiration (no external substrate is present), a sample containing organic matter is injected. The exogenous respiration starts and continues until all the substrate has been removed, and respiration returns to the endogenous rate level.](image)

Figure 3.31 illustrates that exogenous heterotrophic and autotrophic respiration are independent and their respiromgrams can be superimposed. Three respiromgrams are shown, one with only COD addition as acetate showing heterotrophic activity, one with ammonia addition showing nitrifier activity and one with the COD/N mixture.

![Figure 3.31 Illustration of adding together the exogenous heterotrophic and autotrophic respiration rates. The square symbols represent a respirogram with the addition of 20 mg COD L⁻¹ of acetate, the circles represent the respiration rate due to nitrification after the addition of 2.5 mg N L⁻¹ and the line is the respirogram of the mixture of 20 mg COD L⁻¹ and 2.5 mg N L⁻¹ (Kong et al., 1996)](image)

Figure 3.30 shows some typical respiromgrams, some of them only showing the
exogenous respiration rates that, following some respirometric principles, can be directly calculated from the DO data.

The presence of respiration due to nitrification is noteworthy in the respirogram in Figure 3.32. Indeed, when a wastewater sample supplemented with extra ammonium is injected, one can clearly observe additional exogenous respiration due to respiration for nitrification in the absence of nitrification inhibition.

![Figure 3.32 Example of respirograms obtained from BOD tests with wastewater and wastewater mixed with an additional amount of ammonia (Petersen et al., 2002a).](image)

When two experiments are performed, one in the presence of a nitrification inhibitor and one without an inhibitor, one can estimate the concentration of ammonium in the wastewater by making the difference between the BOD₅ of both samples. This oxygen demand divided by 4.33 mg O₂ mg N⁻¹ enables the ammonium concentration in the wastewater sample to be obtained (provided that full nitrification to nitrate has been achieved). Moreover, since ammonification is normally a very quick process, it is not only ammonium that can be quantified like this, but in fact the nitrifiable nitrogen in the wastewater.

Figure 3.33 shows an example of a respirogram for a typical municipal wastewater sample added to biomass without a nitrification inhibitor. In this graph only the exogenous respiration rate is depicted. The interpretation of the respirogram goes as follows (Spanjers and Vanrolleghem, 1995). Starting from the right end of the respirogram, the respiration rate increases gradually until about 50 minutes at which point a sudden decrease in the respiration rate is observed. This sudden decrease is due to the complete removal of ammonium from the activated sludge, after which only hydrolysable organic matter is being oxidized, hence the typical exponential decrease in respiration rate due to the first-order nature of hydrolysis.

![Figure 3.33 An example of respirograms obtained from BOD₅ tests with municipal wastewater (Spanjers and Vanrolleghem, 1995).](image)

The final example presents a respirogram of an industrial wastewater (Figure 3.34).

![Figure 3.34 The results of the OUR test with industrial wastewater depicting three superimposed respirograms corresponding to the degradation of three different solvents (Coen et al., 1998)](image)

Again, only exogenous respiration rates are shown. The respirogram shows in fact the superposition of three respirograms. This could be explained by the presence of three major solvents in the wastewater that were...
degraded in parallel by the acclimated biomass. By making three horizontal lines corresponding to the plateaus, one can delineate the amounts of each of the solvents by taking the corresponding area (see the calculations below). Note that in Figure 3.34 horizontal lines can be used because the measured rates correspond with substrate saturation conditions, whereas in some other cases (for example Figure 3.39) substrate limitation occurs, i.e. the respiration rate decreases as the substrate is exhausted, and sloped lines are used.

3.4.3.2 Calculations

Since BODₘ (mg O₂ L⁻¹) is defined as the amount of oxygen consumed for biodegradation of pollutants, it can be readily calculated by integration of the time series of exogenous respiration rates rO₂,exo (mg O₂ L⁻¹ h⁻¹):

$$\text{BOD}_\text{st} = \int_{t_{\text{pulse}}}^{t_{\text{final}}} r_{O_2,\text{exo}}(t) \cdot dt$$  \hspace{1cm} \text{Eq. 3.20}

Where, t_pulse is the time of pulse addition, and t_final is the time needed to return to the endogenous respiration rate after the sample addition.

Evidently the BODₘ of the injected sample is deduced from:

$$\text{BOD}_\text{st, sample} = \frac{V_{\text{Sludge}} + V_{\text{Sample}}}{V_{\text{Sample}}} \cdot \text{BOD}_\text{st}$$  \hspace{1cm} \text{Eq. 3.21}

Where, V_sample is the volume of sample and V_sludge is the volume of sludge in the test vessel prior to the sample addition.

As explained before in the context of Figure 3.31, the amount of nitrifiable nitrogen (N_nit in mg N L⁻¹) can be calculated from the part of the respirogram that can be attributed to the respiration rate due to nitrification r_{O_2,\text{exo}}^\text{Nit} (mg O₂ L⁻¹ h⁻¹):

$$N_{\text{Nit}} = \frac{1}{4.57 - Y_{\text{ANO}}} \int_{t_{\text{pulse}}}^{t_{\text{final}}} r_{O_2,\text{exo}}^\text{Nit}(t) \cdot dt$$  \hspace{1cm} \text{Eq. 3.22}

Where Y_ANO is the nitrifier's yield coefficient, typically 0.24 mg COD mg N⁻¹.

Similarly, the BODₘ attributed to different fractions of organic matter present in a wastewater can be calculated from the exogenous respiration rates that can be attributed to the superimposed respiration rates r_{O_2,\text{exo}}^i (mg O₂ L⁻¹ h⁻¹), Figure 3.33:

$$\text{BOD}_\text{st}^i = \int_{t_{\text{pulse}}}^{t_{\text{final}}} r_{O_2,\text{exo}}^i(t) \cdot dt$$  \hspace{1cm} \text{Eq. 3.23}

3.4.4 Toxicity and inhibition

3.4.4.1 Purpose

Respirometric techniques have been frequently preferred for the assessment of inhibitory and toxic effects of substances or wastewater on biomass (Volskay and Grady, 1990). Inhibition is the impairment of biological function and is normally reversible. Toxicity is an adverse effect on biological metabolism, normally an irreversible inhibition (Batstone et al., 2002). In this chapter we prefer to use the terms toxicity and toxicant to cover both reversible and irreversible effects. Although toxicity test results are often expressed in terms of IC₅₀ (the concentration which produces 50 % inhibition of the respiration), the IC₅₀ does not give complete insight into the toxic effect of a chemical. In order to predict the effects of the toxic compounds on the removal of organic matter and nutrients or to design mitigative actions in the biological treatment process, the effects of a toxicant on the biodegradation kinetics should be quantified.

3.4.4.2 Test execution

Both decreases in the endogenous and exogenous respiration rates can be used to assess toxicity. If the decrease in the endogenous respiration rate is used as an indicator of toxicity, biomass is first brought to the endogenous state, the toxicant is added and then the reduction in endogenous respiration rate measured. If the toxicant or toxic wastewater is biodegradable, exogenous respiration may occur, interfering with the evaluation of the reduction in the endogenous respiration rate. It has also been found that biomass is less sensitive to toxicants when it is in its endogenous state.

For toxicity assessment based on exogenous respiration rates, respirometers (see Section 3.4.3.1) are the basis. In an exogenous rate-based test, a reference substrate (e.g. acetate for heterotrophic toxicity tests or ammonia for nitrifier toxicity tests) is injected prior to injection of a potentially toxic sample so as to assess the reference respiration rate (i.e. biomass activity). After the toxicant has been added and has had time to affect the biomass, another pulse of reference substrate is injected and the determined activity (e.g. the maximum exogenous respiration rate) is compared to that obtained
prior to the toxicant injection. It is to be noted that the time between the toxicant injection and reference substrate injection may affect the level of toxicity determined since longer-term exposure may lead to a stronger effect or may also lead to biomass adaptation (Figure 3.35). No clear indication is available on optimal exposure times. Sometimes even stimulation of respiration may occur, i.e. the maximum respiration rates for reference substrate degradation after the addition of the toxicant are higher than without the toxicant. This is due to increased energy requirements by the biomass to cope with the toxicant (e.g. energetic uncoupling in the presence of benzoic acid as the toxicant). In any case it is important to always report the applied time between the injections of the toxicant and the reference substrate.

3.4.4.3 Calculations

A toxicity severity level can then be deduced by calculating the ratio in activity levels before and after the toxicant addition.

\[
\text{Toxicity (\%)} = \frac{r_{O2,exo}^{\text{max}}(\text{before}) - r_{O2,exo}^{\text{max}}(\text{after})}{r_{O2,exo}^{\text{max}}(\text{before})} \times 100
\]

Eq. 3.22

Obviously the toxicity severity level will depend on the dose of toxicant applied. Dose-response curves are obtained from a series of experiments in which the toxicity response is measured at different doses, and they allow the assessment of the IC50 (the half maximum inhibitory concentration). Normally the testing protocol to obtain the dose-effect relationship is to perform a reference-toxicant-reference sequence at one dose, replace the biomass with fresh biomass and perform another reference-toxicant-reference sequence at a higher dose. Typically the concentration of the dose is a multiple of the previous dose. This set of reference-toxicant-reference sequences is continued until the biomass is completely inhibited.

To speed up the experimentation the step of replacing the biomass can be omitted (Kong et al., 1994), but then the contact time is not under the control of the experimenter. In Figure 3.36 an example of such fast determination of the dose-effect relationship is given for copper addition to biomass in increasing doses of 0, 2.5, 5.0 and 10.0 ppm. This leads to a copper exposure concentration of 0, 2.5, 7.5 and finally 17.5 ppm, but each with different contact times. The figure clearly demonstrates that the maximum respiration rate for degradation of acetate (the reference substrate in this experiment) decreases rapidly. It is noteworthy that the area under the curve remains the same, i.e. the BOD5 (Section 3.4.3) remains the same since all the acetate is still degraded, albeit at a lower rate. This also means that the respirogram takes longer to complete.

From this experiment the IC50 can be visually deduced to be approximately 7.5 ppm since the maximum respiration rate at this copper concentration is about half the maximum respiration rate in the absence of copper (compare the first, at ~0.5 h, with the third respirogram, at ~1.7 h).
Figure 3.36 Typical respirograms obtained from an ARIKA (Automated Respiration Inhibition Kinetics Analysis) test with copper (Kong et al., 1994). The first respirogram is obtained using pure acetate and the successive respirograms are a series of results where a mixture of acetate with geometrically increasing toxicant concentration (cumulative toxicant concentration: 2.5, 7.5 and 17.5 ppm) was used.

As previously mentioned, autotrophic and heterotrophic respirograms can be superimposed and toxicity testing towards heterotrophic and autotrophic activity can thus be done in a single experiment with a mixture of COD and N as the reference substrate. In Figure 3.37 the impact of two classical toxicants, copper and cyanide, on heterotrophic and autotrophic biomass are assessed. These respirogram series with increasing toxicant concentration show that nitrifiers are more sensitive to cyanide than heterotrophs (IC50 is approximately 0.2 ppm for nitrifiers, whereas it is about 1 ppm for heterotrophs). For copper the IC50 for acetate oxidizers and IC50 for nitrifiers are approximately the same as the whole respirogram decreases together.

### 3.4.4.4 Biodegradable toxicants

So far, only the toxicity of non-biodegradable substances has been considered. However, using respirometry for toxicity determination of biodegradable substances is more involved. When the typical reference-toxicant-reference sequence is used to determine toxicity, a biodegradable toxicant will lead to a respirogram after injection; thus this must be completed before the second reference substrate pulse can be added. If the toxic impact is reversible (i.e. inhibition), it may be that no toxicity can be detected since the toxicant has been removed from the activated sludge. However, contrary to this non-lasting, or temporary, toxicity, lasting toxicity may be assessed with the reference-toxicant-reference sequence, as explained above.

Respirometry can be very useful to study a number of biodegradable toxicants that are inhibitory to their own biodegradation through what is called substrate inhibition, i.e. the substrate on which biomass is growing is inhibitory to the biomass growth process. Nitrification may also be self-inhibited when the ammonia concentration is too high. Respirograms of such self-inhibitory substrates show an increasing respiration rate as the concentration of the biodegradable toxicant decreases with time and its inhibitory effect thus diminishes.

Figure 3.38 illustrates how respirometry can explain complex phenomena occurring in biological wastewater exposed to toxicants (Gernaey et al., 1999).
The use of dynamic models in activated sludge processes has become more and more widespread and has become a way of thinking and communicating about wastewater treatment processes. The Activated Sludge Model No.1 (ASM1) presented by the IAWQ Task Group on Mathematical Modelling for Design and Operation of Biological Wastewater Treatment Processes (Henze et al., 1987) is generally accepted as state-of-the-art, and is used for simulation of wastewater treatment plants in many studies. It will form the basis of this section, which deals with the use of the respiration rate to obtain wastewater fractions in the context of ASM1.

Before establishing the relationship between the respiration rate and the fractions of the ASM1 components, it is essential to explain this model for the heterotrophic process (Table 3.2). It is assumed that the reader comprehends the Gujer matrix as depicted in Table 3.2. In the mass balance of the heterotrophic organisms $X_{OH}$ (component 5, in short: c. 5), the production of $X_{OH}$ by aerobic growth (process, or reaction 1, in short: r. 1) is counteracted by the loss of $X_{OH}$ by heterotrophic decay (r. 4). In this decay process component $X_{OH}$ (c. 5) is converted to component $X_C$ (c. 4). This production of $X_C$ is counteracted by the loss of $X_C$ by hydrolysis (r. 7), leading to production of component $S_B$ (c. 2). $S_S$ is used for heterotrophic growth (r. 1) where it is converted to component $X_{OH}$ (c. 5) at the expense of component oxygen $S_O$ (c. 8), i.e. respiration. A similar reasoning can be made for the processes involving the soluble and particulate nitrogen components ($S_{NH}$, $S_{BN}$ and $X_{BN}$) and autotrophic (nitrifying) organisms ($X_{ANO}$). Anoxic growth (r. 2) with nitrate $S_{NO}$ (c. 9) as the terminal electron acceptor is also considered.

Hence, the wastewater fractions that will be quantified using aerobic or anoxic respirometry are the biodegradable COD fractions: $S_B$, $X_C$, the biomass potentially present in the wastewater: $X_{OH}$ and $X_{ANO}$ and the nitrogen fractions: $S_{NH}$, $S_{BN}$ and $X_{BN}$. Other, inert fractions can be determined as well using respirometric data, but then additional chemical analyses of total and soluble COD and total and soluble nitrogen fractions will be needed. This will not be dealt with here, but can be found in Vanrolleghem et al. (1999) and Petersen et al. (2003).
Table 3.2 Activated Sludge Model No. 1: Gujer matrix (Henze et al., 1987).

<table>
<thead>
<tr>
<th>Component (i)</th>
<th>Process (j)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Process rate ((Q))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anaerobic growth of heterotrophic biomass</td>
<td>$-\frac{1}{V_m}$</td>
<td>1</td>
<td>$-\frac{1-Y_{S_h}}{X_{S_h}}$</td>
<td>$-k_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$-\frac{k_{a,b}}{14}$</td>
<td>$\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
<td>$\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
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<td>$\frac{k_{a,b}}{14}$</td>
<td>$\frac{\Delta t}{14}$</td>
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<tr>
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<td>Anaerobic growth of autotrophic biomass</td>
<td>$-\frac{1}{V_m}$</td>
<td>1</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-k_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{k_{a,b}}{14}$</td>
</tr>
<tr>
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<td>Anaerobic growth of autotrophic biomass</td>
<td>1</td>
<td>$-\frac{4.57-Y_{S_h}}{X_{S_h}}$</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
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<td>$\frac{h_{a,b}}{14}$</td>
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<tr>
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<td>Decay of heterotrophic biomass</td>
<td>$1-f_{a,b}$</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
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<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
</tr>
<tr>
<td>5</td>
<td>Decay of autotrophic biomass</td>
<td>$1-f_{a,b}$</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
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<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
</tr>
<tr>
<td>6</td>
<td>Ammonification of soluble organic nitrogen</td>
<td>1</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
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<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
</tr>
<tr>
<td>7</td>
<td>Hydrolysis of slowly biodegradable substance</td>
<td>1</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
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<td>$-\frac{\Delta t}{14}$</td>
</tr>
<tr>
<td>8</td>
<td>Hydrolysis of organic nitrogen</td>
<td>1</td>
<td>$-\frac{1}{X_{S_h}}$</td>
<td>$-h_{a,b}$</td>
<td>$-\frac{\Delta t}{14}$</td>
<td>$\frac{h_{a,b}}{14}$</td>
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<td>$-\frac{\Delta t}{14}$</td>
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<td>$-\frac{\Delta t}{14}$</td>
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</tbody>
</table>
The total respiration rate of biomass in contact with wastewater is, according to the ASM1:

\[ r_{O2, tot} = \frac{1 - Y_{OH2}}{Y_{ANO}} X_{OH2} \mu_{OH2} + \frac{4.57 - Y_{ANO}}{Y_{ANO}} X_{ANO} \mu_{ANO} \]

Eq. 3.25

Where the specific growth rates \( \mu_{OH2} \) and \( \mu_{ANO} \) are functions of \( S_B \) and \( S_{NHx} \), respectively (Henze et al., 1987).

The concentrations of \( S_B \) and \( S_{NHx} \), in turn, depend on the rates at which \( X_{CB} \), \( S_{N,B} \) and \( X_{CN,B} \) are degraded (Table 3.2). It is clear that all the independent processes summarised in Table 3.2 eventually act on the mass balance of oxygen (and nitrate if the same evaluation is done for anoxic conditions).

There are two approaches for the assessment of model wastewater fractions: direct methods focus on specific fractions that can be directly evaluated from the measured respiration rates (Ekama et al., 1986; Spanjers et al., 1999), whereas optimisation methods use a (more or less simplified) model that is fitted to the measured data (Kappeler and Gujer, 1992; Wanner et al., 1992; Spanjers and Vanrolleghem, 1995). In the latter, numerical techniques are used to find values of the unknown wastewater fractions that lead to the smallest deviation between the model predicted and the measured respiration rates. The optimisation methods for estimation, using numerical techniques, will not be discussed here, but reference is made to Chapter 5.

From the processes described in Table 3.2, it is clear that the total respiration rate is affected by the concentrations of all the biodegradable components and that the cumulative oxygen consumption (i.e. the integral of \( r_{O2} \)) is a measure of the amount of components degraded. Notice that because in direct methods integrals of respiration rates are taken, the measuring frequency and signal-to-noise ratios (measurement error compared to measurement value) are not very critical for the reliable assessment of the component concentrations. This is in contrast to kinetic characterization (Vanrolleghem et al., 1999 and Petersen et al., 2003), where information is obtained from changes in respiration rates (derivative of \( r_{O2} \)). This implies a much higher dependency on the parameter accuracy of the respirometric measurement quality.

Inherent with respirometric tests for wastewater characterization is the use of biomass: the assessment of wastewater components is based on the respirometric response of biomass to wastewater. Two important aspects are associated with the use of biomass. The first aspect is the amount of wastewater component with respect to biomass (\( S_B/X_O \) ratio, Sections 3.4.1.3 and 3.4.3) that is used. Second, in the death-regeneration concept adopted in ASM1, new \( S_B, X_{CB}, S_{NHx}, S_{N,B} \) and \( X_{CN,B} \) are continuously generated from decaying biomass. Within this model it is therefore difficult to distinguish between the components originating from the wastewater and from the biomass itself. In fact the transition between exogenous respiration and endogenous respiration is gradual. The respirometric test should thus be organised in such a way that these rates can be distinguished. This is one of the most challenging problems in respirometric characterization of wastewater in the context of ASM1.

Figure 3.39 shows a respirogram collected in a batch experiment where at the start wastewater is added to endogenous sludge.

In Figure 3.39 only the exogenous respiration rate is presented, i.e. the endogenous respiration is subtracted from the total respiration rate. A typical wastewater respirogram shows an initial peak brought about by the oxidation of readily biodegradable matter, followed by one or more shoulders where successively other components continue to be oxidised. The full area under the respirogram represents the total of biodegradable components (\( S_B + X_{CB} \))/\((1 - Y_{OH2}) + (S_{NHx} + S_{N,B} + X_{CN,B})/(4.57 - Y_{ANO})\), as follows from the above equation on total respiration rate. In Figure 3.39 three
substrate fractions can be discerned, corresponding to $S_0$, $X_{CB}$ and $S_{NHB}$.

### 3.4.5.1 Readily biodegradable substrate ($S_B$)

The readily biodegradable substrate is presumably composed of (simple/low molecular) soluble compounds, such as volatile fatty acids, alcohols, etc. (Henze et al., 1992). The characteristic of these compounds is that they are degraded rapidly and hence provoke a fast respirometric response.

A typical batch test for determination of $S_B$ (e.g. Ekama et al., 1986) involves the addition of a wastewater sample to endogenous sludge, and monitoring the respiration rate until it returns to a level where it can be reasonably assumed that the readily biodegradable substrate is removed from the activated sludge. If other processes occur that are also consuming oxygen (e.g. endogenous and nitrification respiration rates), they must be identified or assumed so that their respiration rates can be subtracted from the total respiration rate (Figure 3.39). Nitrification may be inhibited (Section 3.4.2). Only when the other oxygen consuming processes are accounted for, can the respiration rate related to oxidation of readily biodegradable substrate ($r_{O_2,exo}^{SB}$) be identified and used to calculate the $S_B$ concentration in the wastewater.

The concentration of readily biodegradable substrate initially present in the mixture of biomass and wastewater can then be calculated as follows:

$$ S_B(0) = \frac{1}{1-Y_{OHH}} \left[ \int_0^t r_{O_2,exo}^{SB}(t) \, dt \right] $$  

Eq. 3.26

The concentration of $S_B$ in the wastewater is then easily calculated by taking into account the dilution. The end point $t_{final}$ of the integration interval is the time instant where $S_B$ is completely oxidised and where the exogenous respiration rate for $S_B$ becomes zero. The integral can easily be obtained by determining the area under the curve, for instance by using a spreadsheet program, also known as the graphical method. An alternative to this direct method is the optimisation method, as explained above. This consists of solving the mass balance equations with a numerical integrator to predict the exogenous respiration rates for $S_B$ in such a batch experiment. Depending on the initial value $S_B(0)$ given to the integration algorithm, the simulation will result in a different predicted respirogram. One can therefore search for the $S_B(0)$ value that gives the ‘best fit’ to the measured data. For this simple application the optimisation method may be a bit excessive, but for more complex estimation tasks (next paragraph), the approach becomes more straightforward than direct calculation methods.

Notice that knowledge of the heterotrophic yield coefficient $Y_{OHH}$ is needed for the calculation of $S_B$ from respiration rates. This stoichiometric coefficient is always involved when oxygen consumption is converted to substrate equivalents (see also next section and Sections 3.4.5.2 – 3.4.5.6). The batch test described above is also used to assess other ASM1 components and, using the optimization method, kinetic parameters. This explains its popularity in calibration procedures.

Another batch test (Wentzel et al., 1995) consists of monitoring the respiration rate of unsettled sewage without seed for a relatively long period (up to approximately 20 h). A respirogram similar to the one depicted in Figure 3.40 is obtained. $S_B$ is calculated from the respiration rates observed between the start of the test up to the sudden drop (due to depletion of $S_B$), with correction for the increasing endogenous respiration due to the increase in biomass during the test. In addition to $Y_{OHH}$, knowledge of the net growth rate is required, which can be obtained from the same test.

Ekama et al. (1986) presented a method for determining $S_B$ that involves respiration rate monitoring in a completely mixed reactor operated under a daily cyclic square-wave feed. It is hypothesised that the sudden drop in respiration rate to a lower level, observed upon termination of the feed (Figure 3.41), corresponds...
uniquely to the $S_B$ that has entered via the influent. Hence, the concentration of readily biodegradable substrate in the wastewater can be calculated as:

$$S_B = \frac{V_{max}}{Q_{ww}} \cdot \frac{\Delta r_{O_2,mm}}{1 - Y_{CH0}} \quad \text{Eq. 3.27}$$

**3.4.5.2 Slowly biodegradable substrate ($X_{CB}$)**

It is presumed that slowly biodegradable substrate $X_{CB}$, sometimes also defined as particulate material, is composed of (high-molecular) compounds ranging from soluble to colloidal and particulate (Henze, 1992). The common feature of these compounds is that they cannot pass the cell membrane and must first undergo hydrolysis to low-molecular compounds ($S_B$) that can subsequently be assimilated and oxidized. Because the rate of hydrolysis is lower than the oxidation rate of $S_B$, the respirometric response to $X_{CB}$ is slower and can normally be identified quite easily as a tail to the respirogram.

In a batch test an exponentially decreasing ‘tail’ can frequently be observed in respirograms after the initial SS peak (Figure 3.39). In Figure 3.42, this tailing starts after approx. 0.75 hour. The wastewater concentration of $X_{CB}$ can be assessed in a similar way to the above, using the appropriate $r_{O_2,exo}$ (Kappeler and Gujer, 1992). Simultaneously occurring oxidation processes such as nitrification might interfere with and complicate the identification of the respiration rate governed by hydrolysis. In this case a nitrification inhibitor may be used to facilitate the assessment of $X_{CB}$ (Spanjers and Vanrolleghem, 1995). Alternatively, if the data of such respirometric batch tests are evaluated using the optimisation method to match the response of the model to the data, the nitrification part can easily be extracted from the respirogram (Spanjers and Vanrolleghem, 1995).

The example of Figure 3.43 is presented to stress that all the methods mentioned above using oxygen respiration are also applicable for anoxic respiration tests using nitrate measurements (by lab analysis or using nitrate probes). In this figure not a nitrate respirogram is presented but, in fact, its integral, i.e. the change in nitrate concentration. Backtracking through the different periods with different nitrate utilization rates (the initial period with $S_B$ and $X_{CB}$ biodegradation, followed by a period with biodegradation of $X_{CB}$ only, followed by endogenous nitrate respiration) to the $Y$-axis allows direct assessment of the nitrate used for the different processes and thus calculation of their concomitant concentrations in the wastewater.

The calculation is to be done in a similar way as for the oxygen respiration rate, with some modifications (the respective $\Delta NOx$ of Figure 3.43 substitute the integrals in this case):

$$S_B(0) = \frac{2.86}{1 - Y_{CH0,ax}} \int_{0}^{t} r_{S_B,exo} \, dt \quad \text{Eq. 3.28}$$

$$X_{CB}(0) = \frac{2.86}{1 - Y_{CH0,ax}} \int_{0}^{t} r_{X_{CB},exo} \, dt \quad \text{Eq. 3.29}$$
Where the COD equivalence term of 2.86 g COD g NO₃-N⁻¹ is used. It should be taken into account that the anoxic yield is somewhat lower than the aerobic yield: whereas for \( Y_{OH} \) a value of 0.66 g COD biomass g CODsubstrate⁻¹ is often used, the anoxic yield is suggested to be taken as 0.54 g COD biomass g CODsubstrate⁻¹ (Muller et al., 2003).

### 3.4.5.3 Heterotrophic biomass (\( X_{OH} \))

Some wastewaters can contain significant concentrations of heterotrophic biomass (Henze, 1992), so there is a need to quantify this component. A batch test described by Kappeler and Gujer (1992) and Wentzel et al. (1995) assessed \( X_{OH} \) from the evolution of the respiration rate of raw wastewater without adding a biomass seed. The calculation requires \( Y_{OH} \) and two parameters that can be assessed from the same data: the maximum specific growth rate \( \mu_{OH} \) and the decay coefficient \( b_{OH} \). Respirogramms look like the one presented in Figure 3.40. The procedure basically backtracks the amount of heterotrophic biomass originally present in the wastewater by comparing the original respiration rate with the respiration rate after significant (hence, well quantifiable) growth of \( X_{OH} \).

### 3.4.5.4 Autotrophic (nitrifying) biomass (\( X_{AN} \))

So far, the authors are not aware of procedures in which the autotrophic biomass concentration in wastewater is assessed. This is probably due to the fact that it is quite unlikely to find significant amounts of nitrifiers in the wastewater. However, it could be imagined that a similar procedure as the one developed for \( X_{OH} \) is applicable, i.e. evaluate the respiration rate for nitrification \( r_{O_2,exo} \) of the autotrophs present in the wastewater and compare it to the respiration rate of a culture with known autotrophic biomass concentration \( X_{AN} \), e.g. after significant growth.

### 3.4.5.5 Ammonium (\( S_{NH} \))

The wastewater ammonium concentration can be assessed by using conventional analytical techniques. However, respirometry also offers the possibility to deduce \( S_{NH} \) from batch measurements in a similar way to \( S_B \) and \( X_C \) (provided the test is done with nitrifying activated sludge). As follows from Table 3.2, the nitrifiers yield coefficient \( Y_{AN} \) is needed to convert the oxygen consumption for nitrification to nitrogen concentration by dividing by \( 4.57 - Y_{AN} \). However, the value of \( S_{NH} \) is not very sensitive to \( Y_{AN} \) as \( Y_{AN} \) is small compared to 4.57. Notice that ammonia is also used for assimilation (i.e. about 12 % of the biomass weight as VSS consists of nitrogen), which may require a considerable amount of the nitrogen if a large amount of COD is biodegraded (COD Degraded). The nitrogen that can be nitrified can be approximated by:

\[
N_{NH} = S_{NH} - i_{N,Bio} \cdot Y_{OH} \cdot \text{COD Degraded}
\]

Where \( i_{N,Bio} \) is the nitrogen content of newly formed biomass. From this equation one can easily deduce the original nitrogen concentration when \( \text{COD Degraded} \) and the stoichiometric parameters \( i_{N,Bio} \) and \( Y_{OH} \) are known. Fitting a model in which carbon and nitrogen oxidation are included to the respirometric data (i.e. the optimisation method) will automatically take this correction into account (Spanjers and Vanrolleghem, 1995).

### 3.4.5.6 Organic nitrogen fractions (\( X_{CB,N} \) and \( S_{B,N} \))

Probably because the hydrolysis and ammonification rates of organic nitrogen compounds are relatively fast, little attention has been devoted so far to the establishment of respirometric techniques for \( X_{CB,N} \) and \( S_{B,N} \) quantification (these components have even been abandoned in the subsequent Activated Sludge Models No. 2 and 3). In batch tests, these compounds are typically converted to \( S_{NH} \) before the \( S_{NH} \) that was originally present in the wastewater has been removed by nitrification. These fractions are thus encapsulated in the
determined ammonia concentration. Therefore, $X_{C_{B,N}}$ and $S_{B,N}$ are not directly observable in such tests.

Still, for some wastewaters the hydrolysis and ammonification steps may be considerably slower and quantification of the component concentrations may be required. In such cases, one can imagine a procedure in which the nitrification respiration rate $r_{\text{NO}_2, ex}$ is monitored and interpreted in terms of hydrolysis and ammonification, similar to the way the respiration resulting from COD degradation is interpreted in terms of the hydrolysis process. Subsequently, the amounts of nitrogen containing substrates could be assessed by taking the integral of $r_{\text{NO}_2, ex}$ for the corresponding fractions and dividing these by $(4.57 - Y_{\text{ANO}})$, the stoichiometric coefficient corresponding to nitrification. In case simultaneous COD removal is taking place, correction should again be made for nitrogen assimilated into the new heterotrophic biomass (Section 3.4.5.5).

### 3.5 BIOMASS CHARACTERIZATION

#### 3.5.1 Volatile suspended solids

In biomass characterization tests, activity is usually normalized by expressing a conversion rate per unit of volatile suspended solids (VSS) in order to account for the varying biomass concentrations. Note, however, that VSS is not equivalent to biomass, that is: only a (usually unknown) part of the VSS consists of active biomass, and VSS is only an approximation of the concentration of biomass. In fact, the challenge of the methods described in this section is to assess the part of the VSS that represents the biomass concentration. For further information on how to assess the part of the VSS that is active biomass the reader is referred to Ekama et al. (1996), Still et al. (1996) and Lee et al. (2006).

#### 3.5.2 Specific methanogenic activity (SMA)

##### 3.5.2.1 Purpose

The specific methanogenic activity (SMA) test involves the assessment of the acetoclastic methanogenic activity of a biomass. This is in contrast to the anaerobic biomass activity tests where the overall activity of the biomass, degrading a usually complex substrate, is measured; in these tests the activity is limited by the rate of the slowest degradation step, which is usually the hydrolysis so that the hydrolytic activity of the biomass for that particulate substrate is assessed. The SMA test may be used for monitoring reactor performance or for characterizing biomass prior to its use as an inoculum for the start-up of a new reactor and thus its potential as an inoculum for that specific process (Sorensen and Ahring, 1993).

##### 3.5.2.2 General

In SMA tests, the activity of the methanogens is quantified. This is done by supplying a substrate that can be converted directly into methane. This can be CO$_2$ or H$_2$ gas, but a more common and a more practical substrate is acetate. In literature this has become a standard test as more and more researchers are using it (Ersahin et al., 2014; Jeison and van Lier, 2007). The conversion rate of acetate to methane, normalized to biomass, gives information about the activity of the methanogens in the biomass. It is usually expressed in g COD g VSS$^{-1}$ d$^{-1}$.

To prevent acidification of the anaerobic digestion, VFA production and VFA consumption need to be in equilibrium (Section 3.4.1). The SMA therefore indicates the maximum acetate production during the anaerobic digestion that can be handled by the acetoclastic methanogens without the pH dropping to values that inhibit methanogenesis.

##### 3.5.2.3 Test execution

In SMA tests (like BMP tests), the amount of inoculum (required for anaerobic digestion) is usually measured as volatile suspended solids (VSS), although volatile solids (VS) is also possible (Section 3.4.1).

In an SMA test the ratio between the inoculum and substrate is balanced such that the quantity of methane is high enough to be measured accurately; the flow is to be within the range of the gas flow meter and the concentration of acetate is below inhibition levels. What is often used is an acetate concentration of 2 g COD L$^{-1}$ and the following substrate to inoculum ratio can be used:

$$2 = \frac{VSS_{\text{inoculum}}}{COD_{\text{substrate}}}$$

Both COD and VSS are given in masses supplied to the test, expressed in grams, not concentrations.

- **Stock solutions**
  - The stock solution needed is a phosphate buffer which contains stock solution A ($0.2 \text{ M } K_2HPO_4\cdot3H_2O$: 45.65 g L$^{-1}$) and stock solution B ($0.2 \text{ M } NaH_2PO_4\cdot2H_2O$: 31.20 g L$^{-1}$).
The macronutrient solution contains per litre: 170 g NH₄Cl, 8 g CaCl₂·2H₂O and 9 g MgSO₄·7H₂O.

The micronutrient solution contains per litre: 2 g FeCl₂·4H₂O, 2 g CoCl₂·6H₂O, 0.5 g MnCl₂·4H₂O, 30 mg CuCl₂·2H₂O, 50 mg ZnCl₂, 50 mg HBO₃, 90 mg (NH₄)₆Mo₇O₂·4H₂O, 100 mg Na₂SeO₃·5H₂O, 50 mg NiCl₂·6H₂O, 1 g EDTA, 1 mL HCl 36%, 0.5 g resazurine, and 2 g yeast extract.

**Inoculum**
The inoculum is typical anaerobic sludge from a full-scale biogas plant. Measure total suspended solids (TSS) and total volatile suspended solids (VSS) of the sludge.

**Substrates**
As substrate for aceticlastic methanogenesis activity, sodium acetate-trihydrate salt (NaC₂H₃O₂·3H₂O) (M = 136.02 g mol⁻¹) is used. COD value: 0.4706 g COD per g NaC₂H₃O₂·3H₂O. Typically, a sodium acetate solution (substrate solution) with a concentration of 2.0 g COD L⁻¹ is prepared, which should include the following stock solutions:

- Phosphate buffer: mix 30.5 mL of stock solution A and 19.5 ml of stock solution A (in total 50 mL) per litre substrate medium to obtain a 10 mM phosphate buffer at pH = 7.
- Macronutrients: dose 6 mL per L substrate medium.
- Micronutrients: dose 6 mL per L substrate medium.

Check the pH and measure the COD concentration of the substrate solution. For the blanks, prepare the same solution but without substrate (medium solution).

**Preparation of the reaction vessels and test execution**
Use triplicates for both the blank and sample, i.e. three bottles as blanks (only inoculum and media solutions) and three bottles for the sample (inoculum and substrate solutions). As stated above, an inoculum to substrate ratio of 2:1 (based on VSS) is normally used in the SMA test. Choose the total volume of liquid that is suitable for the reaction vessels. To perform the SMA test, as for the BMP test, the bottles need to be incubated at a desired temperature. The standard mesophilic temperature is 35 °C. The SMA test is continued until biogas production ceases.

**3.5.2.4 Data processing**

The methane produced during an SMA test is measured over time. In Figure 3.44, example data of an SMA test are displayed. Notice that the test did not start at 0 N·mL. This was because the head space of the reaction bottle expanded during the warming up to 35 °C, creating a small amount of gas flow. This gas flow in the first 10 minutes does therefore not represent the actual methane flow. During the first two days a phase of low activity can be observed: the lag phase. This is explained by the fact that the methanogens at t = 0 were introduced into a new matrix where the osmotic pressure, conductivity, nutrient composition or substrate concentrations were different from the matrix where methanogens were taken from (digested sludge). This lag phase is normal for SMA tests. After t = 4 days, the methane production has almost come to a halt. This is most likely because of depletion of the substrate. Prior to the start of an SMA test, the expected methane production, based on the acetate COD, can be calculated. For 1 g COD of acetate, theoretically 350 N·mL of methane can be produced. This can be calculated as follows: 1 mole of acetate yields 1 mole of CH₄, hence 1 g Ac yields 1/59 mole of CH₄. At standard temperature and pressure (STP, that is: 273.15 K and 1013.25 mbar), 1 mole of gas is equivalent to 22.4 L, hence 1 g Ac yields 22.4/59 = 0.380 L CH₄. Since 1 g of Ac represents a COD of 1.085 we get 0.380/1.085 = 0.350 L CH₄ g COD⁻¹.
chosen. In this case the interval between \( t = 2 \) and \( t = 4 \) (in red in Figure 3.44) is chosen because the production rate is the highest here. The maximum production rate of the sludge is 35 N-m\( \text{L} \) d\(^{-1}\). In this example 800 mg VSS of inoculum was introduced and therefore the SMA is 43 N-m\( \text{L} \) g VSS\(^{-1}\) d\(^{-1}\). Note that the most common way of expressing the amount of methane is in COD. One g COD of methane occupies 350 N-m\( \text{L} \) of gas, at STP. Therefore the SMA of this experiment is \( \frac{43}{350} = 0.123 \) g COD g VSS\(^{-1}\) d\(^{-1}\). This value falls within the typical range of 0.1 and 0.2 g COD g VSS\(^{-1}\) d\(^{-1}\) that is commonly found in conventional sludge digesters at WWTPs. Sludge in a UASB-type reactor usually has a higher SMA, between 0.2 and 0.4 g COD g VSS\(^{-1}\) d\(^{-1}\).

- **Recommendations**
  Especially in SMA tests it is important to pay attention to the sensitivity of methanogens to temperature and inhibition by oxygen. For further information on this and some other recommendations the reader is referred to the section on BMP tests.

The way to process the data of a blank group is different from the BMP test, that is: in the SMA test the data of the blank group is not subtracted from the data of the sample group.

### 3.5.3 Specific aerobic and anoxic biomass activity

Kristensen *et al.* (1992) summarized a set of laboratory procedures that have been developed to assess the activity of nitrifiers (AUR: ammonia utilization rate), denitrifying biomass (NUR: nitrate utilization rate) and heterotrophic biomass (OUR: oxygen utilization rate). The three procedures and schematic data examples are presented in Figure 3.45 and discussed below.

#### 3.5.3.1 Maximum specific nitrification rate (AUR)

To assess the ammonia utilization rate (AUR), concentrated biomass (e.g. taken from the return or wastage line) and tap water are mixed in one litre cylinders to reach a suspended solids concentration of 3-4 g VSS\( \text{L}^{-1} \). The activated sludge is kept in suspension by aeration through diffusors, which also provide the biomass with oxygen in a concentration range of 6-8 mg O\(_2\) L\(^{-1}\). After reaching a stable condition (endogenous respiration), ammonia is added to reach an initial NH\(_4\)-N concentration of 20 mg N L\(^{-1}\). Please note that nitrification is an acidifying reaction and pH should be monitored during the test to ensure that no pH effects are occurring. The addition of alkalinity or installing a pH control system with base addition can improve the quality of the AUR determination.

**Figure 3.45** Laboratory respirometric procedures and data examples for characterization of nitrifying biomass (AUR: ammonia utilization rate), denitrifying biomass (NUR: nitrate utilization rate) and heterotrophic biomass (OUR: oxygen utilization rate) (Kristensen *et al.*, 1992).
Samples of 10 mL of activated sludge are then withdrawn at intervals of 15-30 min for 3-4 h. The samples are to be immediately filtered to stop the bio-reactions, and the filtrates can be preserved by addition of 0.1 mL of 4 M H₂SO₄ until the analysis. The samples are subsequently analysed for ammonia nitrogen, and nitrate plus nitrite nitrogen. Alternatively an ion-selective ammonia probe, or otherwise an ion-selective or UV-based nitrate and nitrite sensor, can be used directly in the aerated suspension to have more detailed time series and potentially perform biokinetic modelling studies for the nitrifiers.

The AUR (mg NH₄-N L⁻¹ h⁻¹) is calculated from the slope of the resulting nitrate plus the nitrite production curve and as a control also from the ammonia utilization curve. Indeed, ammonia uptake may also be affected by endogenous heterotrophic activity due to decay and ammonia release heterotrophic growth with concomitant ammonia utilization. The produced oxidized nitrogen forms are directly due to nitrification, of course on the condition that oxygen is sufficiently high at all times to prevent denitrification. The specific AUR (SAUR, mg NH₄-N g⁻¹ VSS h⁻¹) is obtained by dividing the volumetric rate by the biomass concentration (g VSS L⁻¹) set at the beginning of the experiment to be able to compare the nitrifying capacities with typical values.

Nitrification capacity can of course also be deduced from a respirometric experiment with the addition of ammonia (Section 3.4.5.5). The maximum respiration rate that can be attributed to nitrification (r₂O₂,ex) in mg O₂ L⁻¹ h⁻¹, i.e. after subtraction of the endogenous respiration, can be translated into an ammonia conversion rate (mg NH₄-N L⁻¹ h⁻¹) using an equation similar to the one used to obtain the nitrifiable nitrogen (Section 3.4.5.5):

\[
AUR = \frac{r_{\text{ANO,}O₂}}{4.57 - Y_{\text{ANO}}} \quad \text{Eq. 3.32}
\]

Where Yₐₙ₀ is the nitrifier yield coefficient, typically 0.24 mg COD mg N⁻¹.

To separate the activities of the ammonia-oxidizing and the nitrite-oxidizing biomass, it is possible to perform two experiments, one with ammonia addition and one with nitrite addition. The uptake of ammonia and nitrite can be monitored in these separate experiments and translated into the respective activities. Experiments in which nitrite build-up occurs can also be used to extract both activities separately, i.e. by determining the nitrite utilization rate after all the ammonia has been oxidized, one can calculate the activity of the nitrite-oxidizing biomass. The AUR should now be obtained from the ammonia profile, and not from the nitrate profile since the latter is lagging behind the ammonia profile due to the nitrite accumulation in the activated sludge. For this type of analysis to work, it is important that the ammonia-oxidizing capacity is significantly faster than the nitrite-oxidizing capacity because sufficient nitrite must accumulate (more than 2 mg NO₂-N L⁻¹ at ammonia depletion is recommended).

An alternative method for determination of the activities of the two biomass groups involved in nitrification was developed by Surmacz-Gorska et al., (1996). It is based on a respirometric experiment to which first sodium chlorate (NaClO₃, 20 mM, i.e. 2.13 g L⁻¹), an inhibitor for the second step in nitrification, is added when the DO has decreased by about 3 mg L⁻¹, followed by addition of ATU after DO declines with another 2 mg L⁻¹ to inhibit the first step of nitrification. A typical DO concentration profile obtained in a closed bottle test is given in Figure 3.46.

![Figure 3.46 A typical DO concentration profile recorded with the two-step nitrification characterization procedure based on chlorate inhibition of nitrite oxidation (Surmacz-Gorska et al., 1996). The slopes of the DO profile are used to assess the respective f₂O₂ (OURs).](image-url)

The respective respiration rates, expressed in mg O₂ L⁻¹ h⁻¹, are directly obtained from the three DO declines and allow oxygen consumption to be calculated for the two nitrification steps: f₂O₂,NO₂,exo (associated to r₂O₂,NO₂,ex) is calculated from the difference in the DO slope before and after chlorate addition, whereas f₂O₂,NH₄,exo (associated to r₂O₂,NH₄,ex) is obtained from the difference in the DO slope after chlorate addition.
before and after ATU addition. With these respiration rates, the activities of both nitrification biomasses (in mg \( \text{NH}_4\text{-N L}^{-1} \text{ h}^{-1} \) and mg \( \text{NO}_2\text{-N L}^{-1} \text{ h}^{-1} \), respectively) can be calculated as follows:

\[
\begin{align*}
\text{r}_{\text{NH}_4\text{-NO}_2} &= \frac{\text{r}_{\text{AOO, O}_2}}{3.43} - Y_{\text{AOO}} \\
\text{r}_{\text{NO}_2\text{-NO}_3} &= \frac{\text{r}_{\text{NOO, O}_2}}{1.14} - Y_{\text{NOO}}
\end{align*}
\]

Where, \( Y_{\text{AOO}} \) and \( Y_{\text{NOO}} \) are the yield coefficients of the two nitrification steps, typically 0.18 mg COD mg \( \text{NH}_4\text{-N}^{-1} \) and 0.06 mg COD mg \( \text{NO}_2\text{-N}^{-1} \), respectively. Specific activities are again obtained by dividing the volumetric rates by the VSS concentration.

### 3.5.3.2 Maximum specific aerobic heterotrophic respiration rate (OUR)

To determine the oxygen utilization rate related to aerobic heterotrophic activity (OUR), biomass and tap water are mixed to obtain a concentration of suspended solids of 2-3 g VSS L\(^{-1}\) in a batch volume of one litre. An experiment is then conducted with COD in excess. Typically acetate is added in a concentration of typically 200 mg COD L\(^{-1}\), i.e. an \( S_0/X_0 \) ratio of about 1/10 to 1/20. To obtain aerobic heterotrophic endogenous activity, an alternative indicator of aerobic heterotrophic activity, no COD is to be added. Nitrification must be inhibited by the addition of allylthiourea (ATU, typically 5-10 mg L\(^{-1}\)). The biomass is continuously aerated to maintain a DO concentration of 6-8 mg O\(_2\) L\(^{-1}\). In the procedure proposed by Kristensen et al. (1992), the respiration rate is measured by periodically pouring part of the batch into a 300 mL BOD flask to measure the oxygen utilization rate using an oxygen probe introduced into the flask (the LSS principle). OUR can then be calculated from the slope of the resulting DO decline. Note that here the total respiration rate in the presence of a nitrification inhibitor is used as the indicator of aerobic heterotrophic activity, i.e. \( \text{r}_{\text{O}_2, \text{endo}} + \text{r}_{\text{O}_2, \text{ext}} \). The alternative ways of obtaining the respiration rate discussed in Sections 3.2 and 3.3 can of course be applied to obtain OUR.

The specific oxygen utilization rate (SOUR), an often used indicator of biomass activity, is calculated by dividing the OUR by the VSS concentration in the batch experiment.

An important element to consider is that the use of acetate may not be ideal in all cases because some biomasses may have adapted to a feast-or-famine regime and store COD rather than use it directly for growth. The corresponding respiration rate for storage may be quite different than the respiration rate that is preferable for heterotrophic activity assessment. If storage is detected, an alternative COD source should be used for activity assessment, e.g. the previously mentioned BOD reference substrate glutamic acid.

### 3.5.3.3 Maximum specific denitrification rate (NUR)

The specific nitrate utilization rate (SNUR) for denitrification can be assessed by using completely mixed, closed atmosphere, two-litre batch reactors. Concentrated biomass is collected and mixed with tap water in the reactors to obtain a suspended solids concentration of 3-4 g VSS L\(^{-1}\). After reaching a stable condition (endogenous respiration), nitrate is added to obtain an initial concentration of 20-30 mg N L\(^{-1}\). COD, most often as acetate, is added in excess to obtain an initial concentration of 100-150 mg COD L\(^{-1}\). For determination of endogenous NUR, higher concentrations of biomass are to be applied (to reduce the experimentation time) and no COD is added. Since denitrification is a process that increases pH, which is amplified when acetate is the COD source (see pH control results of NUR tests with acetate and glucose in Figure 3.47; Petersen et al., 2002b), the pH should be monitored and corrected for if necessary. Still, the probability of negative pH impacts is much lower than with AUR tests given the lower pH sensitivity of heterotrophic biomass and the lower pH impact of the denitrification.

Samples of 10 mL of activated sludge are withdrawn at intervals of 15-30 min for 3-4 h. Samples are best withdrawn under nitrogen gas addition in order to avoid oxygen intrusion into the reactors. The samples are to be pre-treated as mentioned above for the AUR determination and analysed for nitrate plus nitrite nitrogen. Alternatively, an ion-selective or UV-based nitrate and nitrite sensor can be used in the reactor to have more detailed time series and potentially perform biokinetic modelling studies on the denitrification process. An example of such data is given in Figure 3.47.

The NUR (mg \( \text{NO}_3\text{-N L}^{-1} \text{ h}^{-1} \)) is calculated from the slope of the resulting nitrate plus nitrite utilization curve. The Specific NUR (SNUR, mg \( \text{NO}_3\text{-N g VSS}^{-1} \text{ h}^{-1} \)) is obtained by dividing the volumetric rate by the biomass concentration (g VSS L\(^{-1}\)) set at the beginning of the experiment to be able to compare the denitrifying capacities with typical values.
Anoxic and aerobic heterotrophic activities are closely related as they both reflect the capacity of heterotrophic biomass to oxidize organic matter, with either oxidized nitrogen or oxygen as the electron acceptor. Figure 3.48 shows a typical comparison of NUR and OUR respirometers obtained with the same biomass and acetate addition (Sin and Vanrolleghem, 2004). The tailing-off occurring in both experiments after return to endogenous respiration shows that the aforementioned COD storage occurs both under anoxic and aerobic conditions for this biomass sample.

When comparing respiration rates under anoxic and aerobic conditions, one should be aware that the COD conversion rates are, however, typically lower under anoxic conditions. This is explained in two ways: either the specific conversion rates are lower under anoxic conditions or only a fraction of the total biomass is capable of respiring with nitrate. An often-used biomass characteristic for this phenomenon is the so-called anoxic reduction factor \( \eta \) that makes the ratio between both activities on an electron-equivalent basis (hence the factor 2.86):

\[
\eta = 2.86 \frac{r_{\text{NO}_3, \text{exo}}}{r_{\text{O}_2, \text{exo}}}
\]

Eq. 3.35

The factor \( \eta \) is close to one (~0.85) for readily biodegradable substrate (Ekama et al., 1996). For slowly biodegradable substrate in the activated sludge model No. 1 (ASM1), \( \eta \approx 0.33 \) (van Haandel et al., 1981) and in ASM2, it is 0.66 (Clayton et al., 1991). The reason for this difference has still not been understood.

Use of the ratio \( \eta \) assumes that the growth yield on nitrate or on oxygen is the same. However, the growth yield under anoxic conditions is typically lower than that under aerobic conditions (Muller et al., 2003). This means that for the same COD conversion rate more electron acceptor will be consumed under anoxic conditions (more COD must be burnt to achieve the same biomass production). Therefore this means that, in theory, the reduction factor may be above one if the COD conversion rate is the same.
References


