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ACTIVATED SLUDGE ACTIVITY TESTS

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2.1 INTRODUCTION

Different conditions and factors affect the degree and rate (speed) at which the compounds and contaminants of concern are removed by microbial populations in biological wastewater treatment systems. Certainly, the plant configuration and operational conditions play a major role in the prevalence of specific microbial populations and their activities, but factors as diverse and broad as wastewater characteristics and environmental and climate conditions have a strong influence as well. Eventually, in any biological wastewater treatment system, there will be a need to assess, define and understand the plant performance with regard to the removal of certain contaminants and the response of the sludge to inhibitory or toxic compounds of interest. Moreover, from a modelling perspective it is also of interest to assess and determine the stoichiometry and kinetic rates of the conversion processes performed by specific microbial populations (e.g. ordinary heterotrophic organisms: OHOs; denitrifying ordinary heterotrophic organisms: dOHOs; ammonium-oxidizing organisms: AOOs; nitrite-oxidizing organisms: NOOs; phosphate-accumulating organisms: PAOs; sulphate-reducing bacteria: SRB, also identified as sulphate-reducing organisms, SRO (Corominas *et al.,* 2010); or, anaerobic ammonium-oxidizing organisms: anammox. Thereby, the execution of batch activity tests can be rather useful to: (*i*) study the biodegradability of a given wastewater stream (municipal or industrial), (*ii*) determine the stoichiometric and kinetic parameters involved in the conversion of a specific compound, (*iii*) study the potential interactions (e.g. symbiosis and competition) between microbial populations and (*iv*) assess the potential inhibitory or toxic effects of certain wastewaters, compounds or substances.

The nature and type of the batch activity tests can differ depending upon the compounds of interest and the metabolism and physiology of the microbial populations involved in the removal or conversion processes. For instance, they can range from relatively simple aerobic tests where organic matter removal by OHOs is measured to more complex alternating anaerobic-anoxic-aerobic

batch tests to assess the activity of PAOs under the presence of different electron acceptors (such as nitrate, nitrite and oxygen) from activated sludge systems performing enhanced biological phosphorus removal (EBPR).

This chapter presents an overview of the most common batch activity tests and protocols and their execution with the aim of assessing the conversion processes involved in: (*i*) enhanced biological phosphorus removal by PAOs under alternating anaerobic-aerobic conditions, (*ii*) denitrification via nitrate or nitrite by PAOs, (*iii*) reduction of sulphate by SRBs, (*iv*) removal of organics under aerobic conditions by OHOs, (*v*) denitrification by dOHOs using nitrate or nitrite as final electron acceptor, (*vi*) oxidation of ammonia and nitrite by AOOs and NOOs under aerobic conditions and (*vii*) nitrogen removal by anammox bacteria. These experimental protocols aim to serve as a useful guide that establishes a basis for standardizing batch activity tests for use on existing, emerging and innovative treatment processes. It was decided to start the order of presentation with EBPR systems involving PAOs as the processes are complex and include all three biochemical activated sludge environments: anaerobic, anoxic and aerobic.

Figure 2.1.1 Experimental facilities for activated sludge activity tests at UNESCO-IHE Institute for Water Education in the Netherlands (photo: UNESCO-IHE).

2.2 ENHANCED BIOLOGICAL PHOSPHORUS REMOVAL

2.2.1 Process description

Enhanced biological phosphorus removal (EBPR) can be implemented in activated sludge wastewater treatment systems by introducing an anaerobic stage at the start of the wastewater treatment lines. High P-removal efficiency, lower operational costs, lower sludge production and the potential recovery of phosphorus have contributed to its application and popularity (Mino *et al.,* 1998; Henze *et al.,* 2008; Oehmen *et al.*, 2007). EBPR is performed by phosphorus (polyphosphate)-accumulating organisms (PAOs) (Comeau *et al.,* 1987; Mino *et al.*, 1998) that, by intracellular accumulation of polyphosphate (poly-P), can remove higher quantities of phosphorus (0.35-0.38 g P g VSS^{-1} of PAOs) than OHOs (0.03 g P g VSS^{-1} of OHOs) (Wentzel *et al.*, 2008). The scientific, microbiological and engineering characteristics of the EBPR process have been the main focus of research carried out during the last few decades by different research groups (Wentzel *et al.,* 1986, 1987; Comeau *et al.,* 1986, 1987; Smolders *et al.,* 1994a,b; Mino *et al.,* 1987, 1998; Oehmen *et al*., 2005a, 2005c, 20i306, 2007; Nielsen *et al.,* 2010). In particular, efforts have focused on developing a better understanding of the actual EBPR metabolic mechanisms, to unravel the microbial identity of the organisms involved, and to optimize the required process configurations, all with the aim of improving and increasing the EBPR process efficiency and reliability.

PAOs are heterotrophic organisms. However, unlike OHOs, PAOs have the unique capability of using intracellularly stored poly-P to produce the required energy (adenosine tri-phosphate, ATP) under anaerobic conditions to store readily biodegradable organic matter (RBCOD), such as volatile fatty acids (VFA) like acetate (Ac) and propionate (Pr), as intracellular poly-β-hydroxy-alkanoates (PHAs). Stored PHAs are later utilized under anoxic or aerobic conditions for enhanced phosphorus uptake, glycogen synthesis, biomass growth and maintenance. This feature gives PAOs a competitive advantage over other microbial populations of relevance. Thus, PAOs can be enriched to achieve EBPR by recycling activated sludge through alternating the anaerobic and anoxic or aerobic stages, while directing the influent which is usually rich in VFA to the anaerobic stage. A schematic representation of the PAOs' metabolism is shown in Figure 2.2.1.

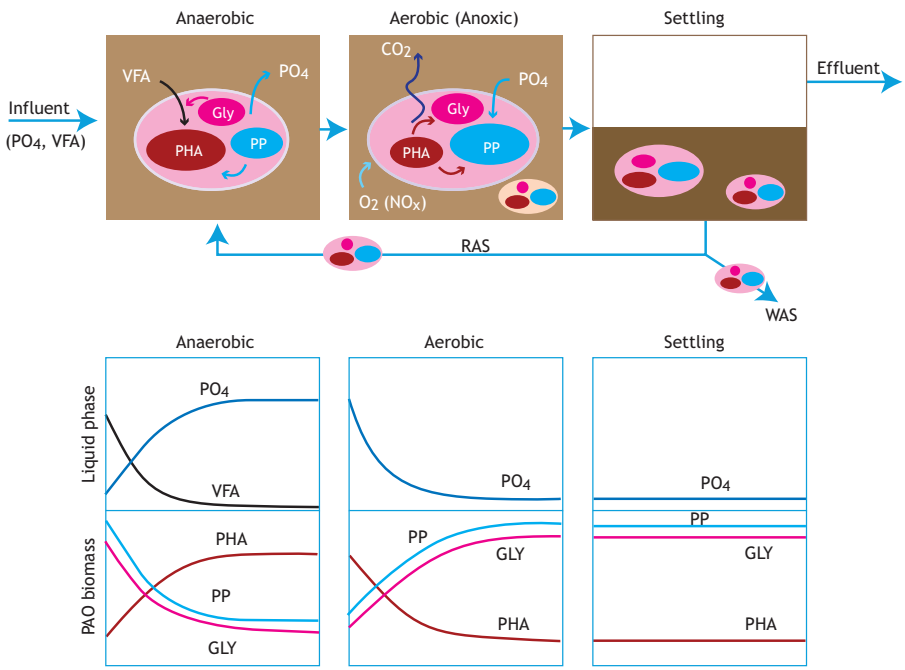

Figure 2.2.1 Conceptual scheme of an activated sludge wastewater treatment plant performing EBPR, illustrating the activity of PAOs (Lopez-Vazquez, 2009; adapted from Meijer, 2004).

In the anaerobic stage, PAOs store intracellularly the readily biodegradable organics present in the raw influent or settled sewage (mostly VFA) as PHAs using two other intracellularly stored polymers that take part in the aforementioned metabolism: poly-P and glycogen (a polymer of glucose). Poly-P is hydrolysed and utilized by PAOs to provide the required energy (as ATP) for the transport and storage of VFA as PHAs (Wentzel *et al.,* 1986), while glycogen is used to supply the required reducing power for the conversion of VFA into PHAs as well as to provide the additional required energy (as ATP) (Comeau *et al.,* 1986, 1987; Smolders *et al*., 1994a; Mino *et al*., 1998). Thus, the anaerobic uptake of VFA by PAOs results in the storage of PHAs and simultaneous hydrolysis of poly-P and glycogen. The most common PHA polymers stored by PAOs are poly-β-hydroxybutyrate (PHB), poly- -hydroxyvalerate (PHV) and poly-β-hydroxy-2-methylvalerate (PH_2MV). Their presence and amount depends on the VFA composition (Ac or Pr). When Ac is the most abundant VFA in the media, PAOs store mostly PHB (up to 90 % of the stored PHAs) (Smolders *et al.,* 1994a), but when Pr is the dominant VFA, then PHAs exist mostly as PHV and PH_2MV (Oehmen *et al.,* 2007).

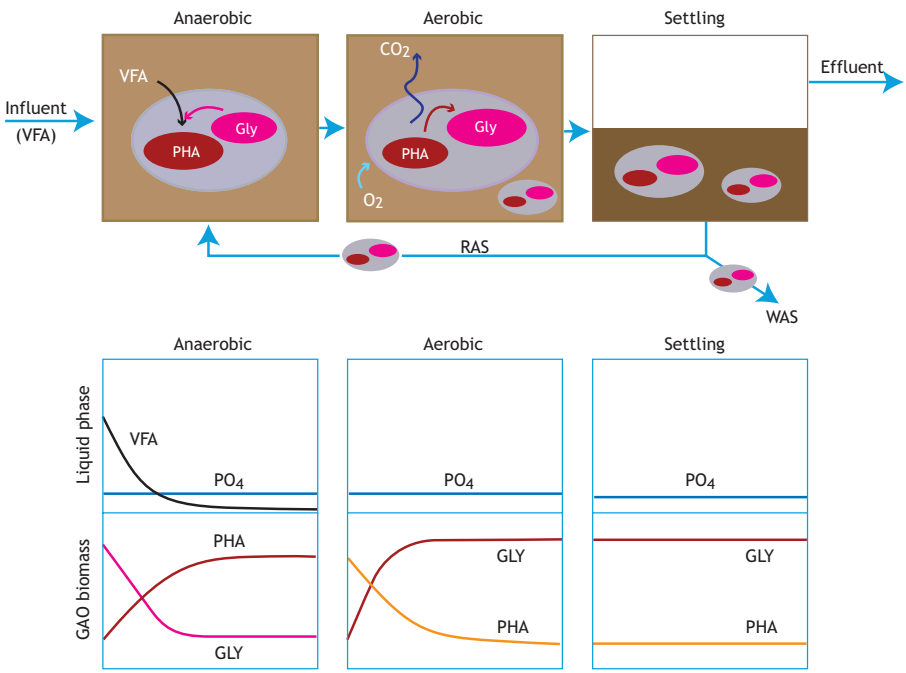

Figure 2.2.2 Conceptual scheme of the microbial activity of GAOs (adapted from Lopez-Vazquez, 2009).

In addition to VFA uptake, the anaerobic hydrolysis of poly-P and glycogen also provides the energy required by PAOs to cover their anaerobic maintenance requirements without carbon uptake. Consequently, the hydrolysis of poly-P leads to the release of orthophosphate (PO_4) into the bulk liquid, which is reflected in an increase in orthophosphate concentration in the liquid phase during the anaerobic stage (Figure 2.2.1). In addition to the uptake of VFA present in the influent of the activated sludge system, PAOs can also store VFA generated by fermentative organisms in the anaerobic stage from fermentable organics present in the influent. Once PAOs reach the aerobic stage, they utilize the PHAs stored in the anaerobic phase as a carbon and energy source using oxygen as the electron acceptor; the energy from this reaction is used to take up and store a higher amount of PO_4 than the amount previously released in the anaerobic stage (Figure 2.2.1). This results in the aerobic uptake and removal of phosphorus from the liquid phase. In the aerobic stage, PHAs are also used to: (*i*) replenish the intracellular glycogen pool, (*ii*) support biomass growth, and (*iii*) cover the aerobic maintenance energy needs of PAOs (Smolders *et al*., 1994b). Net P-removal from wastewater is achieved through the

wastage of activated sludge (WAS) at the end of the aerobic phase, when the sludge contains a high poly-P content (Figure 2.2.1). Alternatively, denitrifying phosphorus-accumulating organisms (DPAOs) exist which can also take up PO_4 under anoxic conditions using nitrate or nitrite as electron acceptors (Vlekke *et al*., 1988; Kuba *et al.,* 1993; Hu *et al.,* 2002; Kerr-Jespersen *et al.,* 1993; Guisasola *et al.,* 2009). Also, PAOs, being heterotrophic organisms, are able to take up carbon sources under aerobic conditions, releasing orthophosphate while the carbon source is available and removing PO_4 afterwards (Guisasola *et al*., 2004; Ahn *et al*., 2007). However, eventually PAO can lose the competition against OHOs due to their metabolic adaptation to permanent aerobic conditions (Pijuan *et al.,* 2006). For a deeper understanding of the metabolism and factors affecting the EBPR process, the reader is referred to materials published elsewhere (Comeau *et al*., 1986; Mino *et al*., 1998; Oehmen *et al*., 2007).

The proliferation of glycogen-accumulating organisms (GAOs) has been observed in EBPR systems under certain conditions (e.g. when acetate or propionate are present as the sole carbon source, when temperatures exceed 20 °C, at pHs below 7.0, and/or at dissolved oxygen (DO) concentrations higher than 2 mg L^{-1}) (Oehmen *et al.,* 2007; Lopez-Vazquez *et al.,* 2009a,b; Carvalheira *et al.,* 2014). GAOs have an apparently similar metabolism to that of PAOs, but they rely solely on their intracellularly-stored glycogen pools as the source of energy and reducing equivalents that drive the anaerobic storage of VFA as PHAs without any contribution from poly-P (Figure 2.2.2). Their presence is often associated with suboptimal EBPR performance because they do not contribute to phosphorus removal, but compete with PAOs for substrate under anaerobic conditions leading to the deterioration of EBPR systems (Saunders *et al.,* 2003; Thomas *et al.,* 2003). Therefore, GAOs are assumed to be an undesirable population in EBPR systems.

2.2.2 Experimental setup

2.2.2.1 Reactors

To assess the EBPR process performance, batch activity tests can be carried out under anaerobic, aerobic and anoxic conditions depending upon the parameters of interest and nature of the study. In any case, the bioreactor(s) used for the execution of tests must: (*i*) avoid oxygen intrusion under anaerobic and anoxic conditions, (*ii*) provide enough aeration capacity to produce DO concentrations higher than 2 mg L^{-1} under aerobic conditions, (*iii*) provide complete mixing conditions, (*iv*) allow temperature control; (*v*) allow pH control, and (*vi*) have ports for sample collection and the addition of influent, solutions, gases and any other liquid media or substrate used in the test (Figures 2.2.4 and 2.2.5).

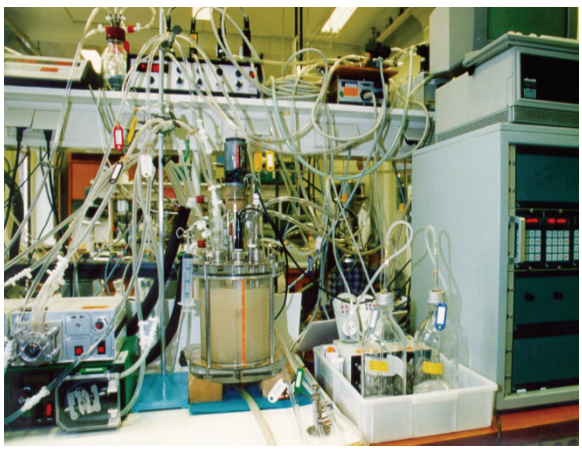

Figure 2.2.3 Vintage EBPR experimental setup with the characteristic yellowish colour of highly enriched biomass with PAOs used at Delft University of Technology in the early 1990s for the development of the TUDelft bio-P metabolic model (Smolders *et al.,* 1994a, 1994b; Murnleitner *et al.,* 1997) and pioneering research on the impact of temperature on EBPR (Brdjanovic *et al.,* 1998a) (photo: Brdjanovic, 1994).

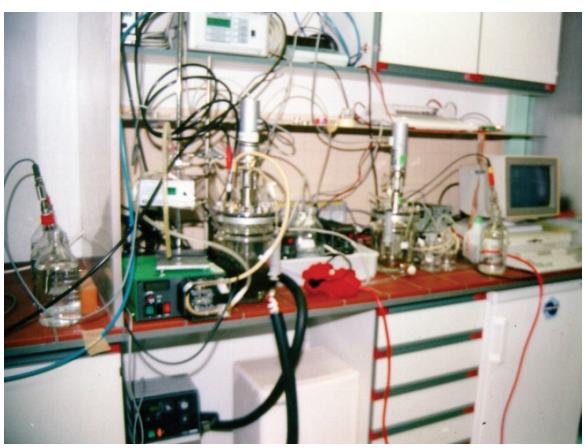

Figure 2.2.4 Temporary experimental setup used to carry out batch tests with activated sludge at the WWTP Haarlem Waarderpolder in the Netherlands. This was the first study (Brdjanovic *et al.,* 2000) in which a separate validation of the TUDelft bio-P metabolic model was carried out using various batch tests with mixed culture biomass from a full-scale plant (photo: Brdjanovic, 1997).

Anaerobic conditions

The experimental setup used for EBPR batch activity tests must be able to create and maintain strict anaerobic conditions. This means that no electron acceptors (namely oxygen, nitrate or nitrite) should be available to the biomass during the anaerobic phase.

A redox probe can be used to monitor the creation of anaerobic conditions when the redox values are lower than -300 mV. The lab setup should be airtight and equipped with an off-gas exit connected to the lid of the bioreactor. Usually, there are three undesirable sources of oxygen: (*i*) the oxygen dissolved in the influent, (*ii*) the residual oxygen present in the activated sludge itself, and (*iii*) oxygen intrusion from the head space. To remove the first two listed sources, N_2 gas should be sparged under mixing conditions from the bottom of the bioreactor for 5 to 10 min prior to the beginning of the test and during influent addition. Sparging time will depend on the mass transfer properties of the gas-liquid interface, which depends on a number of factors including: the dimensions of the bioreactor, the presence and location of baffles, dimensions and stirring speed of mixing blades, gas diffuser configuration and flow rate, and medium composition. To avoid oxygen intrusion, the headspace can be flushed either by the N_2 gas already sparged at the bottom of the bioreactor to the activated sludge or by flushing the head space for 5 to 10 min, depending on the volume of the head space and the gas flow rate. A N_2-gas flow rate of around 30 L h^{-1} is commonly used in lab-scale fermenters with an operating volume of up to 3 L, while a lower flow rate of about 6 L h^{-1} is recommended for batch reactors with working volumes of around 0.5-1.0 L. Sparging N_2 gas from the bottom of the bioreactor is common practice and it can be applied prior to, at the beginning, and during the execution of the activity tests, whereas flushing of the head space is often used during the execution of the test to avoid oxygen intrusion from the atmosphere when mixing the activated sludge. Combining these two approaches is both unusual and unnecessary.

To avoid diffusion of oxygen into the bioreactor, a unidirectional check-valve or a water-lock (containing an oxygen scavenger, such as $NaSO_2$) should be connected to the off-gas line. Alternatively, if the bioreactor is continuously sparged with N_2 gas, resulting in positive pressure inside the bioreactor and a continuous off-gas flow, a check valve or water-lock are not essential for ensuring anaerobic conditions. If N_2 gas is unavailable or cannot be continuously supplied due to limitations of the equipment, the activated sludge should be gently yet completely mixed at slower speeds (much lower than 300 rpm) under airtight conditions until the dissolved oxygen (DO) concentration drops below the detection limit (practically zero) and the redox values are lower than -300 mV. In addition, the volume of headspace should be reduced to minimize the risk of oxygen intrusion by filling the fermenter to the maximum working volume and/or by reducing the surface area of the gas-liquid interface by adding non-reactive, floating polyurethane foam or sponge beads. Silicon rubber stoppers and seals, plastic and aluminium foils, among other materials, are usually used to create airtight conditions.

In addition to avoiding oxygen intrusion, the presence and availability of other electron acceptors (such as nitrate or nitrite) must be prevented to keep strict anaerobic conditions throughout the test. Their prevention is not as straightforward as the removal of oxygen. It often requires an adequate handling of the activated sludge sample prior to the execution of the test. This may involve the controlled addition of nitrifying inhibitors under non-aerated and aerated conditions, or sludge 'washing', as explained later in Section 2.2.3.5).

Anoxic conditions

The creation of strict anaerobic conditions means that no electron acceptors should be present inside the bioreactor, the creation of anoxic conditions indicates that although DO must not be present, other electron acceptors, such as nitrite or nitrate, must be available. This also means that the creation of anoxic conditions must reduce or eliminate oxygen intrusion as is done for anaerobic phases. The experimental setup should allow the (controlled) availability (presence) of the electron acceptors of interest. The desired electron acceptors can be generated either in the system itself, via a preceding nitrification step, or be externally added as nitrate - or nitrite - solutions at defined concentrations at the start of the batch test or during the test. If available, DO concentrations below the detection limit together with redox values in between -200 to 0 mV can indicate that the required anoxic conditions have been reached. Usually, the latter is the most common practice because the experimental configuration can be simplified by doing so, and there is a better control of the required dosing time and concentration. Nevertheless, a combination of several reactors and experimental stages/phases can be made to incorporate a nitrification step between the anaerobic and anoxic phases to provide the required electron acceptors to drive the anoxic metabolism of EBPR cultures (Kuba *et al.,* 1993). When external electron donors are added, the system must have

an adequate dosing port and a way to release the resulting extra pressure created by injecting the liquid volume.

Aerobic conditions

Most commercially available fermenters have gas spargers usually located at the bottom of the fermenter just below the stirring blades of the mixer. When supplying compressed air (e.g. either from a central or a local/portable air compressor), these arrangements can provide a satisfactory oxygen supply leading to DO concentrations reaching far above the limiting conditions of the microbial processes. As a general rule of thumb, DO concentrations of at least 2 mg L^{-1} are considered adequate for most applications. For batch reactors with a working volume of about 3 L, a compressed air flow rate of around 60 L h^{-1} (1 L min^{-1}) can usually provide the required aeration. However, the biomass composition and concentration, wastewater characteristics, organic matter and intracellular PHA content (in the case of EBPR) may increase the DO requirements. Under these conditions, the air flow rate should be increased so as to maintain the DO concentration above 2 mg L^{-1} throughout the test. Alternatively, a pure oxygen supply can be used instead of compressed air to increase the DO availability under specific conditions (e.g. in industrial applications).

In more advanced applications, it may be necessary or desirable to carry out aerobic batch activity tests at a constant (set) DO concentration. For these applications, a two-way DO control can be used to define a DO set point and keep it stable throughout the aerobic batch test. Most advanced fermenters are equipped with such a two-way control operated by at least two solenoids with an on/off function that alternatively supplies air or N_2 gas, depending upon the actual measured DO in the liquid phase.

Less advanced fermenters used for the execution of aerobic batch activity tests can be equipped with a portable air compressor that provides an adequate air flow rate. Aquarium stones can be placed at the bottom of the fermenters in line with the mixing/stirring system to distribute bubbles for good oxygen transfer. As previously discussed, the air supply should be able to produce and sustain a bulk liquid DO concentration of at least 2 mg L^{-1} within the first 10 min.

The two most common commercially available DO probes are the membrane-type and the optical-type. Prior to use (and preferably also after use), they should be calibrated according to the manufacturer's or supplier's instructions. In addition, all connections should be checked. In the case of the membrane-type DO probe, the membrane should be clean, should not have any damage, and the probe should be properly filled with fresh electrolytes. Moreover, no bubbles should accumulate or be trapped on the membrane surface. The surface of optical probes also needs to be cleaned periodically and the head cap replaced annually.

Mixing

Mixing of the bioreactor's content must be generous to favour a homogenous distribution of the activated sludge (mixture of liquid phase and biomass) and wastewater as well as other substances (e.g. orthophosphate, nitrate or nitrite solutions). Commonly, in most 3 L fermenters, a mixing speed of up to 500 rpm can be applied, while slower mixing speeds of around 100 rpm are used in larger fermenters (of 10 L and larger). Excessive mixing can lead to floc breakage, reducing the mass transfer resistance through the flocs and the settling properties. On the other hand, insufficient mixing can result in dead zones, large flocs, sludge stratification, limited diffusion of substrates and oxygen, and, in extreme cases, in settling. Slower mixing speeds can be used as long as the bulk liquid is well mixed and neither stratification nor accumulation of solids is observed. Advanced fermenters can have an automatic control to regulate the mixing speed in time using a vertical axis with blade propellers. Furthermore, the use and installation of vertical blades or baffles connected to the inner side of the fermenter's lid or the choice of a more efficient impeller can further improve the mixing conditions. In less sophisticated systems, mixing can be provided by positioning the fermenters on stirring plates and using magnetic stirrers. As previously mentioned, to reduce the potential oxygen intrusion in anaerobic batch tests, the stirring speed can be reduced as long as it does not compromise the good mixing conditions.

Temperature control

Temperature has a strong effect on the metabolism of PAOs and their competitors (e.g. GAOs) (Brdjanovic *et al.,* 1997; Lopez-Vazquez *et al.,* 2009a,b). Therefore, adequate and stable temperature-controlled conditions are advisable for the execution of the batch activity tests. Advanced fermenters are usually equipped with a double glass wall (double-jacketed reactors) and usually water (of a temperature similar to the target temperature in the bioreactor) is recirculated through the double wall. The water temperature is adjusted in the controlling and operating console of the fermenter (through internal heaters, heat exchangers and condensers) or by using external heating jackets or a water bath and recirculation devices. Depending upon the desired working

temperature, other fluids rather than water can be recommended (e.g. anti-freeze solutions for temperatures lower than 5 ºC or oils for temperatures higher than 30 ºC). Advanced systems can automatically measure the bulk water temperature inside the bioreactor and adjust accordingly to keep a stable temperature. However, it is important to keep in mind that the temperature of the cooling/heating fluid is often different (by a couple of degrees Celsius) from the actual temperature measured in the activated sludge. These differences occurr due to thermal exchange efficiency and thermal exchange between the recirculated fluid and the air during its transport from the controller to the fermenter, in particular when the operating temperature is significantly different from the ambient (room) temperature. Under such conditions, it is recommended to adjust the fluid temperature in the controller unit until the target temperature in the liquid phase is reached and remains stable.

Besides the individual temperature control that a fermenter may have, the entire experimental setup can be located inside a temperature-controlled room set at the target temperature. Nevertheless, if temperature is not an issue and the batch tests can be performed at room or ambient temperature in a defined location, there should be the certainty that the temperature will not fluctuate considerably (less than ± 1-2 ºC) from the preparation until the end of the test. In any case, the temperature of execution must be always recorded and reported.

Last but not least, it is important to mention that both the activated sludge and the wastewater or synthetic medium of the study (whenever applicable) must have the same temperature prior to the execution of the activity tests to avoid temperature shocks and fluctuations that may compromise the outcomes of the tests. Under these circumstances, all the activated sludge, wastewater and solutions need to be exposed to the working temperature and their actual temperature must be monitored until they reach the target temperature. The temperature adjustment of the activated sludge must be carried out with no electron donor present (i.e. no external carbon source). Usually, only a short exposure time of the biomass to the desired temperature of maximum 1 to 2 h is necessary. Whenever needed, the samples could also be acclimatized for longer periods (up to 3-4 h) until the target temperature is reached, but special attention must be paid to avoid compromising the metabolic activity of PAOs (e.g. leading to the consumption of the intracellular compounds). If the temperature difference between the activated sludge and substrate media or wastewater is high or if the temperature effects are of interest (e.g. higher than 5 °C to assess a potential temperature shock), the tests must be conducted as soon as the target temperature is reached.

Usually, most of the tests are executed around 20 °C, but it can be as low as 5 ºC (to assess the biomass activity under winter/cold climate conditions) (Brdjanovic *et al.,* 1997) or as high as 30 - 35 °C for tropical conditions or industrial applications (Cao *et al.,* 2009; Ong *et al.,* 2014), and even up to 55 °C for thermophilic conditions (Lopez-Vazquez *et al.,* 2014). Tests are rarely performed below 5 °C because in practice the temperature of municipal wastewater is seldom colder and is usually around 7 - 12 °C.

pH control

pH is an important operating parameter for EBPR (and many other) processes. This is particularly because the metabolism of PAOs during the anaerobic uptake of carbon (VFA) will result in higher P-release levels at higher pH and lower P release at lower pH (Smolders *et al.,* 1994a). Also, mixing and the vigorous sparging of N_2 gas or compressed air can strip the dissolved CO_2 out of the solution and raise the pH above 7.0. (e.g. in the range of 7.8-8.5), affecting a number of biological and physical processes.

On the other hand, the biological removal of constituents, such as phosphate by PAOs, tends to decrease the buffering capacity of the liquid and change the pH during an experiment. The alkalinity of the wastewater or other solutions added can increase the buffering capacity of the bulk liquid and reduce the fluctuations. CO_2 sparging can compensate for the CO_2 that strips out when mixing or sparging compressed air or N_2 gas. Thus, similar to temperature, pH must be stable prior to, throughout and until the end of the EBPR batch test.

Under certain circumstances, different pH set points can be applied during different experimental phases (e.g. a pH of 7.5 under anaerobic conditions followed by a pH of 7.0 in the aerobic stage). An acceptable fluctuation pH range is assumed to be ± 0.1. In this regard, the use of a two-way pH controller (for acid and base addition, such as HCl and NaOH, respectively) is recommended.

Advanced bioreactor systems usually have pH control settings, but simpler yet reliable external pH controllers can also be used. In less advanced systems, pH levels can be controlled through the manual addition of acid and base solution. The usual molarity of acid and base solutions for pH control is around 0.2 to 0.4 M. If

manual pH control is applied, the concentrations can be lower (e.g. 0.1 M). Depending on the activity of the sludge, different molarities can be used. If the molarity of the solutions is too high, it may lead to sudden pH changes, where the pH values may drop or increase drastically around the set point, crossing the lower or upper limit of the pH control settings and even oscillating below and above the pH set point. Lower molarities may lead to a slow response to adjust the pH to the desired pH set point, which in extreme cases might not be reached and create a considerable dilution of the activated sludge in the bioreactor.

Due to the fast initial speed of microbial conversions, the potential acid or base consumption will be higher at the beginning of the tests or when switching from one phase to another (e.g. from anaerobic to aerobic), but it will usually stabilize by the end of the test. In any case, a considerable deviation from the pH set point (e.g. of more than ± 0.10) must be corrected, preferably within 5-10 sec. The actual pH measured in the liquid phase during the experiment should always be reported.

Certain pH controllers have specific settings that should be adjusted to maintain a stable pH, such as the volume (stroke) of the pulses of acid and base addition and the response time in between acid and base addition pulses. The time in between the acid or base addition pulses should be adjusted to the time that is needed for the system to obtain homogenously mixed conditions after the addition of acid or base.

Similarly to temperature, if pH shocks are to be studied, the EBPR batch activity tests must start as fast as the pH of the activated sludge reaches the target pH of the study. Any required pH adjustment must be performed preferably in less than 5 min prior to the start of the test to avoid any premature or side effects on the metabolism of PAOs (e.g. leading to certain P release or consumption of polymers stored intracellularly). The use of sulphuric acid and alkaline, phosphate buffers and Tris(hydroxymethyl)aminomethane (Tris) solutions must be avoided. This is mostly since they can lead to interferences such as benefiting sulphate-reducing bacteria (SRB) over PAOs (Saad *et al.,* 2013, Rubio-Rincon *et al.,* 2016, submitted), enhancing P precipitation with carbonate species (chemical P-precipitation) (Barat *et al.,* 2008) or increasing the salinity levels beyond those that PAOs can withstand (Welles *et al.,* 2014). It is needless to say that proper pH control is essential for the success of experiments as even very short exposure of biomass to extreme pH (low or high) will quite certainly affect the biomass irreversibly.

All pH meters and sensors should be calibrated immediately (and preferably checked afterwards) according to the manufacturer's or supplier's instructions and all the connections should be checked. Special attention must be paid to the selection and use of pH sensors that can stand the particular characteristics of the wastewater and EBPR sludge subject to study. For instance, high salinity, high chlorides or high H_2S concentrations can lead to interferences if the pH sensors cannot tolerate the higher concentrations. The reader should always verify in manuals, booklets and/or with providers and suppliers if the pH sensors and meters to be used are suitable for the particular wastewater characteristics to be tested.

Sampling and dosing ports

The reactors/fermenters used to carry out the batch activity tests should also have conveniently located sampling and dosing ports to ensure the collection of representative samples from the liquid phase as well as favour a fast dispersion or mixture of any substance or solution added to the bulk of the liquid. The sampling ports can be composed of flexible (rubber or plastic) tubing with an inner diameter that makes it possible to connect different syringes of 5, 10 or 20 mL volume, but also of smaller or bigger volumes (e.g. of 1 or even 50 mL). The sampling port inside the fermenter needs to reach a favourable depth and location to allow a satisfactory sampling before, during and at the end of the test. Usually, the sampling port can be located at the middle level of the lowest third or quarter of the bioreactor's working volume subject to the provision of well-mixing conditions. The most important requirement is to obtain a representative sample from a well-mixed bioreactor.

Regarding the dosing ports, they need to be located and positioned in such a way that they allow a fast dispersion of the solutions or substances added into the bioreactor. They can be well defined injection ports located on the lid of the bioreactor or flexible openings (e.g. through the septum). A formal and structured location and integration of the sampling ports into the fermenter configuration is mostly required when working with airtight reactors to avoid oxygen intrusion. Moreover, the use of lab clips (or similar devices) is recommended to close the tubing of the sampling ports or temporarily unused dosing ports and avoid spillages and splashes caused by a possible increase in the internal pressure in the bioreactor. To counteract any potential under or overpressure, a needle can be inserted into a septum located on the lid of the bioreactor and a tedlar bag (or a similar flexible container filled with an inter gas

- e.g. nitrogen) can be connected to the needle to connect the gas phase of the headspace with the inert gas.

2.2.2.2 Activated sludge sample collection

Contrary to some wastewater treatment processes, the sampling time and location of an EBPR activated sludge sample is highly dependent on the type of batch activity test to be conducted. The latter is based on the alternating anaerobic-(anoxic)-aerobic conditions required by the physiology of PAOs. Thus, a fresh sample should preferably be collected at the end of the preceding reaction stage. Thereby, for an anaerobic batch test, the activated sludge sample should be collected at the end of the aerobic phase at the full- or pilot-scale wastewater treatment plant or 'parent' laboratory bioreactor, whereas, for an aerobic batch test, the sample can be collected at the end of the anaerobic or anoxic phase depending on the system configuration. For the execution of anoxic tests, the sludge can be collected at the end of the anaerobic stage. Alternatively, samples collected in the aerobic phase can be used to execute sequential anaerobic-aerobic, anaerobic-anoxic or anaerobic-anoxic-aerobic batch tests.

Certainly, the sampling location will depend on the system configuration. In full- and pilot-scale wastewater treatment plants, the physical borders between stages must be identified prior to sampling. In extreme cases, where the phases are not (physically) well defined, the redox limits or boundaries need to be determined with the use of a DO meter, redox meter and/or by determination of the nitrate and nitrite concentrations. In lab-scale systems (usually operated on a time-base mode), the sample collection can be relatively easier, since the reaction time defines the length of the stages. To obtain homogenous and representative samples, the sludge samples must be collected in sampling spots where well-mixed conditions take place. Ideally, batch activity tests must be performed as soon as possible after collection (in less than 1-2 h for tests to be conducted with sludge collected at the end of an aeration tank/phase or in a few minutes (2-3 min) for sludge collected at the end of an anaerobic or anoxic phase). In lab-scale systems, in principle, this should not be a problem if the batch activity tests are performed in the same laboratory and their execution is coordinated and synchronized with the operation cycle of the lab bioreactor. Also, at full- and pilot-scale treatment plants, batch activity tests can be performed *in situ* shortly after the collection of activated sludge if the sewage plant laboratory is conditioned and equipped with the required experimental and analytical equipment (Figure 2.2.4).

If the batch activity tests cannot be performed *in situ* on the same day, an activated sludge sample can be collected at the end of the aerobic stage. Afterwards, the sampling bucket can be properly stored and transported in a fridge or in ice (below or close to 4 °C) under non-aerated conditions and the activity tests should be performed not later than 24 h after sampling. The sampling collection at the end of the aerobic stage and storage under non-aerated conditions at the lower temperature can help to preserve the original biomass condition by slowing down the bacterial metabolism. Therefore, in principle it is advised not to aerate the activated sludge samples since this could lead to P release and oxidation of intracellular compounds (such as PHAs, glycogen and even poly-P). This also implies that the biomass present in the activated sludge sample needs to be 're-activated' and acclimatized to the target pH and temperature of interest prior to the execution of the batch activity tests. In any case, the *in situ* execution of the batch activity tests is preferable. The total volume of activated sludge to be collected depends on the number of tests, bioreactor volume and total volume of samples to be collected to assess the biomass activity. Often 10-20 L of activated sludge from full-scale wastewater treatment plants can be collected. On the other hand, samples collected from lab-scale reactors rarely contain more than 1 L because lab-scale systems are usually smaller (from 0.5 to 2.2 L and under certain cases up to 8-10 L) and the maximum volume that can be withdrawn from lab-scale reactors is often set by the daily withdrawal of the excess of sludge from the system (which is directly related to the applied sludge retention time (SRT) and, consequently, defined by the growth rate of the organisms).

2.2.2.3 Activated sludge sample preparation

For batch activity tests performed *in situ*, in principle, the sludge will be merely transferred from the parent bioreactor (in the case of a lab-enriched sludge) or reaction tank (in the case of pilot-scale or full-scale plants) to the fermenter or bioreactor where the activity tests will take place. Usually, the sludge transfer must take place before the end of the reaction phase that precedes the reaction phase of interest. Then, the batch activity test can start as soon as the desired pH, redox conditions and temperature are adjusted. During the adjustment until the test starts, the same or similar conditions to those prevailing when the sludge samples

were collected should be kept. This means that sludge samples collected at the end of the anaerobic stage should be kept under anaerobic conditions and therefore must not be aerated or exposed to the presence of any electron acceptor. Similarly, samples collected in the aerobic stage must be aerated and sludge samples collected in the anoxic stage should not be aerated. If desirable, a few milligrams of nitrate can be added to activated sludge samples collected in the anoxic tanks (to a final concentration of ~5 mg NO_3-N L^{-1}) to maintain anoxic conditions as long as it is necessary.

If only EBPR tests will be executed and nitrification tests are not of interest, then a nitrification inhibitor can be added to the sludge sample immediately after the sludge is transferred to the fermenter (e.g. Allyl-N-thiourea: ATU to a final concentration of 20 mg L^{-1}). This will restrain nitrification and consequently (*i*) avoid higher oxygen consumption in aerobic EBPR batch tests and, (*ii*) limit the accumulation of nitrate (or nitrite) if samples are aerated prior to the execution of anaerobic tests. Should the real and actual conditions be assessed, then the corresponding batch activity tests must be conducted right away after sludge collection with the minimum adjustments and stable conditions required (e.g. for pH and temperature). A comprehensive sampling procedure must be carried out before, during, and after the tests to document the results obtained. However, in addition, the execution of batch activity tests under favourable conditions to PAOs is always recommended. This can help to (*i*) assess the EBPR potential that the system can have, (*ii*) benchmark the EBPR plant activity, (*ii*) detect interferences, and (*iv*) contribute to the definition of improvement strategies.

As described elsewhere, interferences to PAOs can be (but are not limited to) the presence of nitrate or nitrite in the aerobic sludge samples collected to execute anaerobic batch tests, the existence of RBCOD in anaerobic samples for the performance of aerobic or anoxic tests, or the detection of nitrite in anoxic samples intended to carry out aerobic batch tests. Thus, if tests are designed to be conducted under favourable conditions to PAOs then such interferences should be avoided. After this, sludge samples can be exposed shortly (for 1 or maximum 2 h) to a pre-treatment or preparation step as a troubleshooting strategy. For instance, to remove the nitrate present in an aerobic sample intended for an anaerobic batch test (~5-10 mg NO_3-N L^{-1}), after collection the sludge can be transferred to the airtight batch bioreactor and gently mixed under non-aerated conditions. The nitrate (and nitrite) concentration can be monitored until it drops below the detection limits. Rapid detection techniques, such as nitrate and/or nitrite detection paper strips (e.g. Sigma-Aldrich), can be rather useful here. Once nitrate is no longer observed, the corresponding anaerobic batch test can start. If RBCOD is detected in a sample taken from an anaerobic tank, the anaerobic conditions can be extended after the sludge is transferred to the airtight bioreactor until no more RBCOD is observed, before the anoxic or aerobic test starts. Anoxic samples to carry out aerobic tests where nitrate is observed do not need pre-treatment since nitrate is innocuous to PAOs under aerobic conditions. However, if nitrite is detected, it must be removed because it has been proven to be rather inhibitory and even toxic to PAOs under certain aerobic conditions (Pijuan *et al.,* 2010; Zhou *et al.,* 2012; Yoshida *et al.,* 2006; Saito *et al.,* 2004; Zeng *et al.,* 2014). To avoid nitrite, a similar approach like the one previously described for the removal of nitrate can be applied.

When the batch tests cannot be performed *in situ* and sludge samples are stored under cold conditions (at around 4 °C), sludge samples need to be 're-activated' because the cold temperature considerably slows down the bacterial metabolism. Due to the particular physiology of PAOs, to reactivate the sludge it must be aerated for 1-2 h at the pH and, particularly, at the temperature of execution of the batch activity tests. This procedure will help to remove residual biodegradable organics. If only EBPR activity tests will be carried out, a nitrification inhibitor should be added (e.g. ATU at a final concentration in the bioreactor of 20 mg L^{-1}) prior to aeration. If a potential interference is detected (e.g. nitrate, nitrite or RBCOD) prior to the 1-2 h aeration period, the sludge reactivation can start with a pre-treatment step at the temperature and pH of interest. Afterwards, even if the objective is only to assess the anoxic or aerobic activity of PAOs, the EBPR activity tests should start with an anaerobic incubation stage using synthetic or real wastewater as a feed. This practice will ensure that the PAOs will have PHAs intracellularly stored to carry out their aerobic or anoxic metabolisms.

Nevertheless, in general, the objective of the experimental plan should be to minimize as much as possible the needs for transport, cooling, storage and reactivation of the sludge (among other potential steps). Whenever possible, it is best to always use 'fresh' sludge (and substrate/media).

2.2.2.4 Substrate

When real wastewater (either raw or settled) is used for the execution of activity tests, it can be fed in a relatively

straightforward manner to the bioreactor/fermenter. For normal (regular) conditions, the feeding step takes place at the beginning of the anaerobic stage to favour the VFA uptake by PAOs and the intracellular availability of PHAs. If judged necessary, a rough filtration step (using 10 µm pore size filters) can be used to remove the remaining debris and large particles present in the raw wastewater. If the activity tests need to be performed at different biomass concentrations, the treated effluent from the plant can be collected and used for dilution (assuming that solids effluent concentrations are relatively low, e.g. 20-30 mg TSS L^{-1}). If different carbon or phosphorus sources and concentrations are to be studied, the plant effluent can also be used to prepare a semi-synthetic media containing a RBCOD concentration of between 50 and 100 mg COD L^{-1}.

Batch activity tests are frequently performed with synthetic wastewater: (*i*) to ensure a better control of the experimental conditions, (*ii*) to create the desired redox conditions, (*iii*) to study and assess the effects of different wastewater composition, or (*iv*) to evaluate the inhibitory or toxic effects of certain solutions or compounds. However, such practice can be expensive due to the potentially large amount of chemicals needed.

Depending upon the nature, purpose and sequence of the activity tests (anaerobic, anoxic or aerobic), the carbon and phosphorus concentrations present in a synthetic wastewater can vary since they are usually the subject of removal and study. Moreover, the concentrations may be adjusted proportionally to the length or duration of the test. Usually, concentrations of up to 50 and 100 mg RBCOD L^{-1} are used when activated sludge samples are obtained from full-scale plants and of up to 400 mg L^{-1} in the case of lab-enriched cultures (though higher concentrations are sometimes applied). Regarding the phosphorus concentrations, synthetic solutions can contain none or low P concentrations when assessing the anaerobic P release or up to 100-120 mg PO_4-P L^{-1} when testing the maximum aerobic P-uptake activities. However, regardless of the nature of the activity test, synthetic wastewater must contain the required macro- and micro-elements (especially potassium and magnesium but also calcium, iron, zinc, cobalt, among others) in sufficient amounts and suitable species that PAOs require in order to avoid any metabolic limitation that may jeopardize the outcomes of the batch activity tests (Brdjanovic *et al.,* 1996). A suggested synthetic wastewater recipe for an initial orthophosphate concentration of 20 mg P L^{-1} can contain per litre (Smolders *et al.,* 1994a): 107 mg NH_4Cl, 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). Overall, it is important to underline that the minimal concentrations of K and Mg need to be proportional to the phosphorus concentration following a molar 1:1:3 Mg:K:P ratio. This is mostly because Mg and K are essential for poly-P formation since they serve as counter-ions in poly-P. If desired, the synthetic wastewater can be concentrated, sterilized in an autoclave (for 1 h at 110 ºC) and used as a stock solution if several tests will be performed in a defined period of time. However, the solution must be discarded if any precipitation or loss of transparency is observed.

For experiments performed with lab-enriched cultures, it is best to execute the tests with the same (synthetic) wastewater used for the cultivation according to the carbon or phosphorus concentrations of the study. Alternatively and similar to full-scale samples, the effluent from the bioreactor can be collected, filtered through rough pore size filters to remove any particles, and used to prepare the required media with the desired carbon and phosphorus concentrations for the execution of the activity tests.

For the execution of (conventional) anoxic tests, nitrate and nitrite solutions can be prepared to create the required anoxic redox conditions. For this purpose, different stock solutions can be prepared using nitrate salts and nitrite salts. However, for practical applications, it is recommended to follow a step-wise approach and carefully monitor their addition so their concentrations in the bulk water do not exceed more than 10 mg L^{-1} in the case of nitrite and 20 mg L^{-1} for nitrate. This will avoid a potential inhibitory effect due to nitrate or nitrite accumulation as described elsewhere (Saito *et al.,* 2004; Yoshida *et al.,* 2006; Pijuan *et al.,* 2010, Zhou *et al.,* 2007, 2012). Moreover, pH levels lower than 7.0, in combination with higher nitrite concentrations, can be comparatively more inhibiting to PAOs because nitrite can be present as free nitrous acid (FNA) - the 'protonated' species of nitrite. At pH 7.0, Zhou *et al.* (2007) and Pijuan *et al.* (2010) observed 50 % inhibition of the anoxic and aerobic metabolism of PAOs at FNA concentrations of 0.01 and 0.0005 mg HNO_2-N L^{-1}, respectively (equivalent to 45 and 2 mg NO_2-N L^{-1} at pH 7.0). Therefore to ensure their availability during the execution of the anoxic tests, the nitrite or nitrate concentrations need to be monitored during the tests and depending on their concentrations, nitrite or nitrate solutions will need to be added in different steps.

When tests are conducted to assess the potential inhibitory or toxic effects of given compounds at different concentrations, concentrated stock solutions can be prepared and added during the test at the concentrations of interest. Tests performed to assess whether the inhibitory or toxic effects are reversible must be carried out after 'washing' the biomass to remove the inhibiting or toxic compound(s). The washing step is often performed by consecutive settling and re-suspension of the sludge sample in a carbon-free media (either fully synthetic or using a treated effluent after filtration) under the redox conditions of interest. Similarly, when maintenance tests need to be executed, a carbon- and phosphorus-free media can be used at the redox conditions and operating conditions of the study.

2.2.2.5 Analytical procedures

Most of the analytical procedures required (for the determination of total P, PO_4, NH_4, NO_2, NO_3, MLSS, MLVSS, among others) should be performed following standardized and commonly applied analytical protocols detailed in Standard Methods (APHA *et al.,* 2012). VFA determination (for acetate, propionate and even other volatile fatty acids) can be conducted by gas chromatography (GC), high pressure liquid chromatography (HPLC) or by applying regular analytical determination protocols. However, contrary to most of the analytical parameters of interest, the determination of PHAs and glycogen requires a more demanding sample preparation and sophisticated equipment and procedures, and in addition, their determination procedures are only to be found in specialized scientific literature. Therefore, the analytical procedures for the determination of PHAs and glycogen are described in more detail in the following paragraphs in this chapter.

- **PHA**

As mentioned earlier, the most common PHA polymers stored by PAOs are poly-β-hydroxy-butyrate (PHB), poly-β-hydroxy-valerate (PHV) and poly-β-hydroxy-2-methyl-valerate (PH_2MV). Their relative presence and stored amount depends on the VFA composition (Ac or Pr) and the type of metabolism involved in the storage (PAO or GAO metabolism). For enriched lab cultures performing EBPR (where PAOs are the dominant organisms composed of more than 90 % of the total biomass), and when Ac is the most abundant VFA, PAOs store VFA mostly as PHB (up to 90 % of PHAs) (Smolders *et al.,* 1994a). However, when Pr is the dominant VFA, then PHV and PH_2MV can be composed of up to 45 % and 53 % of the total PHAs stored, respectively (Oehmen *et al.,* 2005c). When GAOs are present in EBPR systems, the sludge stores higher amounts of PHV too. For instance, lab-enriched GAO cultures (comprising more than 90 % of the total biomass) cultivated with Ac as VFA leads to a PHB and PHV accumulation of around 73 % and 26 %, respectively (Zeng *et al.,* 2003a, Lopez-Vazquez *et al.,* 2007, 2009a), whereas an enriched PAO culture cultivated under similar conditions contains mostly PHB and less than 10 % PHV (Smolders *et al.,* 1994a). Meanwhile, GAO lab-systems fed with Pr result in practically no PHB accumulation, but up to 43 % PHV and 54 % PH_2MV (Oehmen *et al.,* 2006). It is important to underline that the PHA analytical determination technique provides the PHA contents of the MLSS quite accurately. This implies that a precise determination of MLSS is equally important to obtain correct PHA concentrations and to accurately determine net conversions during a biochemical stage.

Compared with full-scale EBPR systems, the determination of PHAs in lab-scale enriched EBPR systems is usually easier since lab-scale systems are smaller and, more importantly, EBPR cultures are enriched with PAOs (> 90 %) (Oehmen *et al.,* 2004, 2006; Lopez-Vazquez *et al.,* 2007) and consequently, the intracellular PHA contents can reach up to 10 % of the total MLSS concentration depending upon the VFA type available (Lopez-Vazquez *et al.,* 2009a). On the other hand, in the best case, the PHA contents accumulated in the mixed biomass from full-scale systems reach between 1 and 2 % of the total MLSS concentration because PAOs (and GAOs) hardly comprise more than 15 % of the total bacterial population (Lopez-Vazquez *et al.,* 2008a). This implies that the analytical determination of PHAs from full-scale samples may not always be suitable, reliable or therefore representative of the direct collection of grab or composite samples. In extreme cases, PHA contents may fall below the detection limit. From an economic perspective and in view of the required resources (in terms of analytical equipment, costs of chemical consumables and highly qualified lab staff), the PHAs determination will probably not be (cost) effective when performed on samples from a full-scale plant. Alternatively, to assess the potential accumulation of PHAs in full-scale systems, real full-scale sludge samples can be used to execute batch activity tests under more favourable and controlled conditions for EBPR that can maximize accumulation of PHAs and facilitate its analytical determination (Lanham *et al.,* 2014). Nevertheless, this latter approach still requires the

analytical determination of PHAs to be performed with high precision.

Regarding the analytical determination of the different PHA polymers, it has been a matter of discussion and improvement since the late 1990s (Baetens *et al.,* 2002). So far, the most reliable method involves two slightly different procedures (Oehmen *et al.,* 2005b): (*i*) one for the determination of PHB and PHV polymers, and (*ii*) another for the determination of PHV and PH_2MV.

For both determination procedures, activated sludge samples must be collected *in situ* in (15 mL) centrifugation tubes. The sample volume should be sufficient to obtain around 20 mg of TSS. To preserve the sample, 4-5 drops of paraformaldehyde (37 % concentration) have to be added to the plastic centrifugation tube (in a fume hood) prior to collection of the sample, and once the sample is taken, it should be stored temporarily at 0-4 °C for around 2 h. To remove the remaining paraformaldehyde and dissolved solids in the liquid phase, samples need to be washed twice with tap water. The washing steps include: (*i*) centrifugation (for 10 min at 4,500 rpm), (*ii*) careful withdrawal of the supernatant by decanting (if a solid pellet is formed) or otherwise with a pipette, avoiding the removal of any particle or solid, (*iii*) tap water addition (10 mL), and (*iv*) re-suspension with a vortex. After the second washing step, the sample must be centrifuged one more time, and supernatant must be discarded. Afterwards, the sample must be stored at -20 °C and subsequently freeze-dried in a lyophilizer at -80 °C and 0.1 mbar for 48 h (or longer), until the sample is fully dried. Once the sample has been freeze-dried, the digestion, esterification and extraction procedures can start.

As described by Oehmen *et al.* (2005b), for PHB and PHV determination, 20 mg of the freeze-dried sample can be transferred to a digestion tube and added to 2 mL of an acidified methanol solution containing a 3 % sulphuric acid (H_2SO_4) concentration and approximately 100 mg L^{-1} of sodium benzoate. Afterwards, samples are digested and esterified for 2 h at 100 °C. After digestion and esterification, samples are cooled down to room temperature; distilled water is added and mixed vigorously. 1 h of settling time must be provided to achieve a phase separation. The chloroform phase can be transferred to a vial, dried with 0.5-1.0 g of granular sodium sulphate pellets and separated from the solid phase. Standard solutions can be prepared in parallel at defined concentrations using commercial co-polymers of R-3-hydroxybutyric acid (3HB) and R-3-hydroxyvaleric acid (3HV) copolymer (7:3). After extraction and esterification, 3 µL of the liquid phase can be injected into a chromatograph. Certain recommended characteristics and operating conditions of the chromatograph are: (*i*) to be equipped with a DB-5 column (30 m length × 0.25 mm I.D. × 0.25 µm film), (*ii*) to apply a 1:15 split injection ratio, (*iii*) to use helium (He) as the carrier gas at a flow rate of 1.5 mL min^{-1}, (*iv*) to be equipped with a flame ionization detector (FID) operated at 300 °C with an injection port at 250 °C, and, (*v*) to vary the oven temperature starting at 80 °C for 1 min, increasing 10 °C min^{-1} up to 120 °C, and then to further increase it at a temperature pace of 45 °C min^{-1} up to 270 °C , and hold it at 270 °C for 3 min. When following this procedure and conditions, the PHB and PHV peaks will show up around 2 and 3 min after injection.

Alternatively, for PHB and PHV determination, another procedure followed by Smolders *et al.* (1994a) involves the addition of 20 mg of freeze-dried biomass to 1.5 mL of dichloroethane, and 1.5 mL of concentrated HCl as well as 1-propanol 1:4 (in volume). 1 mg of benzoic acid in the 1-propanol solution is added as internal standard. Samples are digested and esterified for 2 h at 100 °C and, at least every 30 min, samples are vortexed. After cooling, 3 mL of distilled water is added. The contents are mixed vigorously on a vortex and afterwards centrifuged for a few minutes to obtain a satisfactory and well-defined phase separation. About 1 mL of the lower (organic) phase is drawn off and filtered over a small column of dried water-free sodium sulphate into GC sample vials. As a recommendation, 3 standards must be run for every series of 15 samples. When using this method, 1 µL of the lower liquid phase from the solution can be injected into a gas chromatograph equipped and operated as follows: (*i*) using a HP Innowax column (30 m length × 0.32 mm I.D. × 0.25 µm film), (*ii*) applying a 1:10 split injection ratio, (*iii*) using He as the carrier gas (at a flow rate of 6.3 mL min^{-1}), (*iv*) operating a FID at 250 °C, applying an injection temperature of 200 °C, (*v*) with an initial oven temperature of 80 °C kept for 1 min, that increases to 130 °C at temperature pace of 25 °C min^{-1}, and then to 210 °C at 15 °C min^{-1} and finally held at 210 °C for 12 min. This long final time is recommended to elute propylesters of no interest (e.g. from cell wall constituents). Last but not least, the PHA contents of the biomass is reported as a percentage of the MLSS concentrations, which is used to calculate the PHA concentrations.

If the activated sludge samples contain high concentrations of salts, a saline washing solution with a similar osmotic strength like that of the original sample should be used instead of tap water. This will avoid the cytolysis of the cells and preserve the intracellular compounds (such as PHAs and glycogen) avoiding their dissolution and potential loss through the supernatant. However, when a saline washing solution is used, the high concentration of total dissolved solids (TDS) in the remaining liquid (after centrifugation) may precipitate and lead to apparent deviations in the MLSS concentrations of the original sample, from which the PHA contents will be determined. To compensate, a correction factor will be needed to take into account the potential effect of the TDS on the final solids sample when the PHA concentrations are determined.

Although the two previous analytical procedures can be rather accurate for the determination of PHB and PHV, none of them can, without any further modification, be satisfactorily used for the determination of PH_2MV (of particular importance when propionate is present as a carbon source in EBPR cultures). Thus, to improve the PH_2MV extraction, Oehmen *et al.* (2005b) recommend applying the same procedure described for PHB and PHV determination, but using an acidified methanol solution containing 10 % H_2SO_4 (instead of 3 % H_2SO_4) and extending the digestion phase at 100 °C to 20 h. Since a commercial product to be used as a direct standard for PH_2MV determination is not available, Oehmen *et al.* (2005b) recommended the use of 2-hydroxycaproic acid which is assumed to have a similar relative response to that of PH_2MV (based on the fact that these two molecules are isomers of each other). This procedure has proven useful for the simultaneous determination of PHV and PH_2MV, but not for PHB. As a consequence, if the three polymers (PHB, PHV and PH_2MV) must be determined, the two different determination procedures must be performed. Further details about the analytical PHA determination techniques can be found in the original sources (Baetens *et al.,* 2002; Oehmen *et al.,* 2005b). From a microscopic visualization perspective, Nile blue A stain can be used to qualitatively visualize PHAs and Neisser stain for poly-P (Mino *et al*., 1998; Mesquita *et al*., 2013). Further details about the microscopic observation of these and other intracellular polymers and the use of different stains can be found in Chapter 7 on Microscopy.

- **Glycogen**

EBPR cultures utilize glycogen as a source of energy and reducing power for the storage of PHAs. Glycogen ($C_6H_{10}O_5$) is a multi-branched polysaccharide of glucose ($C_6H_{12}O_6$) similar to starch and cellulose but with a different glycosidic bond and geometry between molecules (Dircks *et al.,* 2001; Wentzel *et al.,* 2008). Its relative presence and intracellular storage by EBPR cultures depends on the VFA composition (Ac or Pr), influent P/C ratio, and dominant organisms (either PAOs or GAOs) (Schuler and Jenkins, 2003; Oehmen *et al.,* 2007). In enriched lab PAO cultures cultivated with Ac as carbon source (where PAOs compose of more than 90 % of total biomass), at influent P/C ratio lower than 0.04 mol mol^{-1}, the glycogen fractions can reach up to 20 % of the total MLVSS concentration, whereas at influent P/C ratios higher than 0.04, the glycogen fractions are usually lower than 15 % (Smolders *et al.,* 1995; Schuler and Jenkins, 2003; Welles *et al.,* 2016, submitted). Similarly, lab-enriched PAOs cultures cultivated with Pr as carbon source tend to store less intracellular glycogen that often does not reach more than 15 % MLVSS since PAOs' anaerobic metabolism on Pr requires less glycogen hydrolysis for anaerobic P release and intracellular PHA storage (Oehmen *et al.,* 2005c). Conversely, GAO cultures enriched in the laboratory using Ac or Pr as carbon source can have glycogen fractions as high as 30 % MLVSS regardless of the carbon source fed (Filipe *et al.,* 2001b; Zeng *et al*., 2003a; Oehmen *et al.,* 2005a,c; Dai *et al.,* 2007; Lopez-Vazquez *et al.,* 2009a). Similar to PHA determination, the determination of the intracellular glycogen content may be easier to estimate in lab-scale systems (where EBPR cultures can comprise more than 90 % of the total microbial population) but it will not be so straightforward in full-scale systems since PAOs (and GAOs) hardly comprise more than 15 % of the total bacterial population (Lopez-Vazquez *et al.,* 2008a). Consequently, the glycogen fractions present in full-scale EBPR systems may hardly reach more than 5 % of the total MLVSS concentrations, which makes its determination more difficult and challenging when compared to lab-scale systems. Nevertheless, it may be still feasible but it requires analytical determination of high precision (Lanham *et al.,* 2014). Glycogen ($C_6H_{10}O_5$) is a multi-branched polysaccharide; it should be hydrolysed and extracted prior to its determination. Thus, different methods have been proposed for the analytical determination of glycogen, ranging from enzymatic hydrolysis tests (Parrou and Francois, 1997) to biochemically-based (Brdjanovic *et al.,* 1997) and through its indirect determination by high-performance liquid chromatography (HPLC) as glucose after an acid hydrolysis and extraction (Smolders *et al.,* 1994a; Lanham *et al.,* 2012). Unfortunately, a direct method is not yet available. For practical reasons and after several

improvements throughout the years, the HPLC method after acid hydrolysis and extraction is one of the most frequently applied procedures. The HPLC method, after acid hydrolysis and extraction for the determination of glycogen, consists of the digestion of an activated sludge sample diluted with 6 M HCl, leading to a final HCl concentration of 0.6 M HCl, and digested at 100 °C for 5 h. After digestion, the sample is allowed to cool down to room temperature under quiescent conditions and the supernatant is filtered through 0.2 or 0.45 µm pore size filters. The filtered supernatant is poured into a vial and glycogen can be quantified by HPLC as glucose (Smolders *et al.,* 1994a). The latter is because glycogen ($C_6H_{10}O_5$) shares the same carbon content as glucose ($C_6H_{12}O_6$) (on a carbon mole basis). However, the determination of glycogen as glucose content extracted from the biomass is not entirely accurate as the non-glycogen glucose-containing content of the biomass (cells) will also make a part of the extracted material, and the glycogen of other glycogen-containing populations beside EBPR (e.g. GAOs) will do the same too. Recently Lanham *et al*. (2012) improved the glycogen extraction technique. Freeze-dried samples prepared like those for PHA determination can be used: activated sludge can be collected *in situ*, added to a 15 mL centrifugation tube containing 4-5 drops of formaldehyde (37 % concentrated), stored at 0-4 °C for around 2 h, washed with tap water and freeze-dried. They recommend using a ratio of 1 mg freeze-dried sludge to 1 mL 0.9 M HCl solution to improve the acid hydrolysis and extraction of glycogen for its further determination as glucose. Then, depending upon the sludge aggregation, the sludge samples can be digested for 2 h in the case of flocculant sludge, 5 h for granular sludge and 3 h if the aggregation state is not known or if it varies. Later on, 5 mg of the freeze-dried sample can be added to 5 mL of a 0.9 M HCl solution, digested for 5 h at 100 °C, supernatant filtered through 0.2 µm pore size filters and measured as glucose by HPLC. If the latter procedure is applied, then the 5 mg of the freeze-dried sample should be carefully and precisely weighed and the results will be reported as a percentage of MLSS. The previous HPLC determination technique has proven to be sufficiently accurate and reliable in lab-enriched cultures where EBPR populations are dominant. However, as previously discussed, their determination in sludge samples from full-scale systems may not be accurate enough. Tentatively, Periodic Acid-Schiff (PAS) stain can be used to get a rough microscopic qualitative estimation of glycogen and other carbohydrate granules present in the cells (Mesquita *et al*., 2013).

2.2.2.6 Parameters of interest

To determine and assess the metabolic activities of PAOs, different stoichiometric ratios and kinetic rates for the anaerobic, anoxic and aerobic stages can be estimated based on the data collected from the execution of the batch activity tests. Table 2.2.1 shows a description of the expected parameters of interest.

2.2.3 EBPR batch activity tests: preparation

This section describes not only the different steps but also the apparatus characteristics and materials needed for the execution of the batch activity tests.

2.2.3.1 Apparatus

1. An (airtight) batch bioreactor or fermenter equipped with a mixing system and adequate sampling ports (as described in Section 2.2.2.1).
2. A nitrogen gas supply (recommended).
3. An oxygen supply (compressed air or pure oxygen sources).
4. A pH electrode (if not included/incorporated in the batch bioreactor setup).
5. A 2-way pH controller via HCl and NaOH addition (alternatively a one-way control - generally for HCl addition - or manual pH control can be applied through the manual addition of HCl and NaOH).
6. A thermometer (recommended temperature working range of 0 to 40 °C).
7. A temperature control system (if not included in the batch bioreactor setup).
8. A DO meter with an electrode (if not included/incorporated in the batch bioreactor setup).
9. An automatic 2-way dissolved oxygen controller via nitrogen and oxygen gas supplies (if not included in the batch bioreactor setup and if tests must be performed at a defined dissolved oxygen concentration).
10. Confirm that all electrodes and meters (pH, temperature and DO) are calibrated less than 24 h before execution of the batch activity tests in accordance with the guidelines and recommendations from the manufacturers and/or suppliers.
11. A centrifuge with a working volume capacity of at least 250 mL to carry out the sludge washing procedure (if required).
12. A stop watch.

Table 2.2.1 Stoichiometric and kinetic parameters of interest for activated sludge samples performing EBPR.

Parameter	Symbol	Typical unit on a mole basis	Typical unit on a mg or g basis
ANAEROBIC PARAMETERS			
Stoichiometric			
Anaerobic orthophosphate release to VFA uptake ratio	$Y_{VFA\_PO4,An}$	P-mol C-mol^{-1}	mg P mg VFA^{-1}
Anaerobic glycogen utilization to VFA uptake ratio	$Y_{Gly/\ VFA,An}$	C-mol C-mol^{-1}	mg C mg VFA^{-1}
Anaerobic PHA production to VFA uptake ratio	$Y_{VFA\_PHA,An}$	C-mol C-mol^{-1}	mg C mg VFA^{-1}
Anaerobic PHB formation to VFA uptake ratio	$Y_{VFA\_PHB,An}$	C-mol C-mol^{-1}	mg C mg VFA^{-1}
Anaerobic PHV formation to VFA uptake ratio	$Y_{VFA\_PHV,An}$	C-mol C-mol^{-1}	mg C mg VFA^{-1}
Anaerobic PH_2MV formation to VFA uptake ratio	$Y_{VFA\_PH2MV,An}$	C-mol C-mol^{-1}	mg C mg VFA^{-1}
Anaerobic PHV formation to PHB formation ratio	$Y_{PHV/PHB,An}$	C-mol C-mol^{-1}	mg C mg C^{-1}
Kinetic			
Maximum specific anaerobic VFA uptake rate	$q_{VFA,An}$	C-mol C-mol^{-1} h^{-1}	mg VFA mg active biomass^{-1} h^{-1}
Maximum specific anaerobic PO_4 release rate	$q_{PP\_PO4,An}$	P-mol C-mol^{-1} h^{-1}	mg P mg active biomass^{-1} h^{-1}
Maximum specific anaerobic PHA production rate	$q_{VFA\_PHA,An}$	C-mol C-mol^{-1} h^{-1}	mg PHA mg active biomass^{-1} h^{-1}
Anaerobic PO_4 release maintenance rate	$m_{PP\_PO4,An}$	P-mol C-mol^{-1} h^{-1}	mg P mg active biomass^{-1} h^{-1}
Anaerobic ATP maintenance coefficient	$m_{ATP,An}$	mol ATP C-mol^{-1} h^{-1}	mg ATP mg active biomass^{-1} h^{-1}
Anaerobic secondary PO_4 release rate	$m_{PP\_PO4,Sec,An}$	P-mol C-mol^{-1} h^{-1}	mg P mg active biomass^{-1} h^{-1}
ANOXIC PARAMETERS			
Stoichiometric			
Anoxic PHA degradation to NO_X consumption ratio	$Y_{NOx\_PHA,Ax}$	C-mol N-mol^{-1}	mg C mg NO_X^{-1}
Anoxic glycogen formation to NO_X consumption ratio	$Y_{NOx\_Gly,Ax}$	C-mol N-mol^{-1}	mg C mg NO_X^{-1}
Anoxic poly-P formation to NO_X consumption ratio	$Y_{NOx\_PP,Ax}$	P-mol N-mol^{-1}	mg P mg NO_X^{-1}
Anoxic biomass growth to NO_X consumption ratio	$Y_{NOx,Bio,Ax}$	C-mol N-mol^{-1}	mg C mg NO_x^{-1}
Anoxic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ax}$	C-mol C-mol^{-1}	mg C mg C^{-1}
Anoxic poly-P formation to PHA consumption ratio	$Y_{PHA\_PP,Ax}$	P-mol C-mol^{-1}	mg P mg C^{-1}
Anoxic biomass growth to PHA consumption ratio	$Y_{PHA\_Bio,Ax}$	C-mol C-mol^{-1}	mg C mg C^{-1}
Kinetic			
Maximum specific anoxic PHA degradation rate	$q_{PHA,Ax}$	C-mol C-mol^{-1} h^{-1}	mg PHA mg active biomass^{-1} h^{-1}
Maximum specific anoxic glycogen formation rate	$q_{PHA\_Gly,Ax}$	C-mol C-mol^{-1} h^{-1}	mg Gly mg active biomass^{-1} h^{-1}
Maximum specific anoxic poly-P formation rate	$q_{PO4\_PP,Ax}$	P-mol C-mol^{-1} h^{-1}	mg PP mg active biomass^{-1} h^{-1}
Maximum specific anoxic biomass growth rate	$q_{Bio,\ Ax}$	C-mol C-mol^{-1} h^{-1}	mg active biomass mg active biomass^{-1} h^{-1}
Anoxic ATP maintenance coefficient	$m_{ATP,Ax}$	mol ATP C-mol^{-1} h^{-1}	mg ATP mg active biomass^{-1} h^{-1}
Anoxic endogenous respiration rate	m_{NOx}	N-mol C-mol^{-1} h^{-1}	mg NOx mg active biomass^{-1} h^{-1}
AEROBIC PARAMETERS			
Stoichiometric			
Aerobic PHA degradation to O_2 consumption ratio	Y_{PHA}	C-mol mol O_2^{-1}	mg C mg O_2^{-1}
Aerobic Glycogen formation to O_2 consumption ratio	Y_{Gly}	C-mol mol O_2^{-1}	mg C mg O_2^{-1}
Aerobic Poly-P formation to O_2 consumption ratio	Y_{PP}	P-mol mol O_2^{-1}	mg P mg O_2^{-1}
Aerobic PAO biomass growth to O_2 consumption ratio	Y_{PAO}	C-mol mol O_2^{-1}	mg C mg O_2^{-1}
Aerobic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ox}$	C-mol C-mol^{-1}	mg C mg C^{-1}
Aerobic Poly-P formation to PHA consumption ratio	$Y_{PHA\_PP,Ox}$	P-mol C-mol^{-1}	mg P mg C^{-1}
Aerobic biomass growth to PHA consumption ratio	$Y_{PHA\_Bio,Ox}$	C-mol C-mol^{-1}	mg C mg C^{-1}
Kinetic			
Maximum specific aerobic PHA degradation rate	$q_{PHA,Ox}$	C-mol C-mol^{-1} h^{-1}	mg PHA mg active biomass^{-1} h^{-1}
Maximum specific aerobic glycogen formation rate	$q_{PHA\_Gly,Ox}$	C-mol C-mol^{-1} h^{-1}	mg Gly mg active biomass^{-1} h^{-1}
Maximum specific aerobic poly-P formation rate	$q_{PO4\_PP,Ox}$	P-mol C-mol^{-1} h^{-1}	mg PP mg active biomass^{-1} h^{-1}
Maximum specific aerobic biomass growth rate	$q_{Bio,\ Ox}$	C-mol C-mol^{-1} h^{-1}	mg active biomass mg active biomass^{-1} h^{-1}
Aerobic ATP maintenance coefficient	$m_{ATP,Ox}$	mol ATP C-mol^{-1} h^{-1}	mg ATP mg active biomass^{-1} h^{-1}
Aerobic endogenous respiration rate of a culture	m_{O2}	mol O_2 C-mol^{-1} h^{-1}	mg O_2 mg active biomass^{-1} h^{-1}

2.2.3.2 Materials

1. Two graduated cylinders of 1 or 2 L (depending upon the sludge volumes used) to hold the activated sludge and wash the sludge if required.
2. At least 2 plastic syringes (preferably of 20 mL or at least of 10 mL volume) for the collection and determination of soluble compounds (after filtration).
3. At least 3 plastic syringes (preferably of 20 mL) for the collection of solids, particulate or intracellular compounds (without filtration).
4. 0.45 µm pore size filters. Preferably not of cellulose-acetate because they may release traces of cellulose or acetate into the collected water samples. Consider having at least twice as many filters as the number of samples that need to be filtered for the determination of soluble compounds.
5. 10 or 20 mL transparent plastic cups to collect the samples for the determination of soluble compounds (e.g. soluble COD, acetate, propionate, orthophosphate, nitrate, nitrite).
6. 10 or 20 mL transparent plastic cups to collect the samples for the determination of mixed liquor suspended solids and volatile suspended solids (MLSS and MLVSS, respectively). Consider the collection of these samples by triplicate due to the variability of the analytical technique.
7. 15 mL plastic tubes for centrifugation for the determination of PHAs and/or glycogen.
8. A plastic box or dry ice box filled with ice with the required volume to temporarily store (for up to 1-2 h after the conclusion of the batch activity test) the plastic cups and plastic tubes for centrifugation after the collection of the samples.
9. Plastic gloves and safety glasses.
10. Pasteur or plastic pipettes for HCl and/or NaOH addition (when pH control is carried out manually).
11. Metallic lab clips or clamps to close the tubing used as a sampling port when samples are not collected from the bioreactor/fermenter.

2.2.3.3 Media preparation

- **Real wastewater**

  If real wastewater will be used to carry out the batch activity test, the sample needs to be collected at the influent of the corresponding wastewater treatment plant and the batch activity test performed as soon as possible after collection. Depending on the nature of the test, the researcher should decide whether to take a sample of raw sewage or settled sewage (if the plant employs primary settling). If due to location, transportation issues or other logistics, tests cannot be performed in less than 1 or 2 h immediately after collection, then one should keep the wastewater sample cold until the test is conducted (e.g. by placing the bucket or jerry can in a fridge at 4 °C). Nevertheless, prior to the execution of the test, the temperature of the wastewater needs to be adjusted to the target temperature at which the batch activity test will be executed (preferably reached in less than 1 h). A water bath or a temperature-controlled room can be used for this purpose, as described in Section 2.2.2.1.

- **Synthetic influent media or substrate**

  If tests can be or are desired to be performed with synthetic wastewater, depending on the type of tests (anaerobic, anoxic or aerobic), the synthetic influent media can contain a mixture of carbon and orthophosphate sources plus necessary (macro and micro) nutrients. Generally, they can be mixed all together in the same media (for anaerobic-(anoxic)-aerobic tests); split in two solutions (*i*) C source and (*ii*) P source (plus nutrient solution); or prepared separately if they need to be added in different phases or time. The usual compositions and concentrations are:

  a. Carbon source solution: This is usually composed of a RBCOD source, preferably volatile fatty acids such as acetate or propionate, depending on the nature or goal of the test and the corresponding research questions. Sometimes, more complex substrates are used, containing a mixture of RBCOD and slowly biodegradable COD (SBCOD); however, these are not applied in the tests described in this chapter, and thus are omitted. For anaerobic batch activity tests, the COD concentration in the feed needs to be set to a level that ensures that all the COD is consumed within the anaerobic stage. For batch activity tests performed with activated sludge from a full-scale plant, usually COD concentrations not higher than 100 mg L^{-1} are recommended. For lab-scale activated sludge samples, the COD concentrations can be as high as the influent COD concentration of the lab-scale system (and even sometimes 2 to 3 times higher) as long as the RBCOD fed is fully removed in the anaerobic stage and is not toxic or inhibitory to PAOs.
  b. Orthophosphate source solution: The orthophosphate concentrations can be adjusted as desired depending on the purpose of the experiment. For single anaerobic batch test experiments only, orthophosphate concentrations

can be as low as 2-3 mg PO_4-P L^{-1} or even be excluded, whereas concentrations as high as 75 mg PO_4-P L^{-1} for full-scale samples or more than 120 mg PO_4-P L^{-1} for lab-enriched cultures (Wentzel *et al.,* 1987) can be added to assess the maximum P-uptake capacity of sludge under anoxic or aerobic conditions (when an anaerobic phase precedes the anoxic or aerobic test).

c. The nutrient solution: This should contain all the required macro (ammonium, magnesium, sulphate, calcium, potassium) and micronutrients (iron, boron, copper, manganese, molybdate, zinc, iodine, cobalt) to ensure that cells are not limited by their absence and avoid obtaining wrong results and, in extreme cases, the failure of the test. Thus, despite the fact that their concentrations may seem very low, it is necessary to make sure that all of the constituents are added to the solution in the required amounts. The following composition (amounts per litre of nutrient solution) is recommended (based on Smolders *et al.,* 1994a): 107 mg NH_4Cl, 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). Similar nutrient solutions can be used as long as they contain all the previously reported required nutrients.

- **Nitrate or nitrite solution**

  When the batch tests comprise an anoxic stage, nitrate or nitrite solutions (as required) can be used to create the anoxic conditions in the batch activity tests (Section 2.2.2.4). Nitrate and nitrite salts can be used for this purpose (e.g. KNO_3 or $NaNO_2$, respectively). Nevertheless, their addition must be carefully monitored to ensure their presence and availability without creating any inhibitory or even toxic effect on the biomass. Thus, it is recommended to keep their concentrations below 20 mg NO_3-N L^{-1} and 10 mg NO_2-N L^{-1} (at pH 7.0).

- **Washing media**

  If the sludge sample must be 'washed' to remove an undesirable compound (which may be even inhibitory or toxic), it is necessary to prepare a nutrient solution to wash the sludge that contains per litre (Smolders *et al.,* 1994a): 107 mg NH_4Cl, 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). The washing process can be repeated twice or three times. Afterwards, the following preparation steps of the batch activity tests can be performed. In special cases when sludge from a full-scale plant is used, plant effluent may be used for washing purposes (given that its composition allows for this).

- **Formaldehyde solution**

  To prepare and preserve the samples for the analytical determination of PHAs and glycogen, a commercial formaldehyde solution (37 % concentration) is needed.

- **ATU (Allyl-N-thiourea) solution**

  To inhibit nitrification, an ATU solution can be prepared to reach an initial concentration of around 20 mg L^{-1} (after addition to the sludge). The ATU solution must be added before the sludge is exposed to any aerobic conditions (including the sludge sample preparation or acclimatization).

- **Acid and base solutions**

  These should be 100-250 mL of 0.2 M HCl and 100-250 mL 0.2 M NaOH solutions for automatic or manual pH control, and 10-50 mL of 1 M HCl and 10-50 mL 1 M NaOH solutions for initial pH adjustment if the desired operational pH is very different from the pKa value of the buffering agent.
- The working and stock solutions required to carry out the determination of the analytical parameters of interest must be also prepared in accordance with Standard Methods (APHA *et al.,* 2012) and the corresponding protocols.
- It is recommended to take a sample of the media prior to execution of the experiment to confirm/check the initial (desired) concentration of the parameter(s) of interest (e.g. COD, orthophosphate, etc.).

2.2.3.4 Material preparation

- The number of samples (and their volume) to be collected should be defined in accordance with the type of analysis, the volumes required for the analytical determination and the number of replicates of the dissolved parameters of interest:

a. E.g. a 3 mL sample needs to be collected if this is the minimum volume required to determine the acetate concentration in that sample by duplicate.
b. When two or more parameters will be determined using the same collected sample, then the required volumes must be summed up: e.g. if 3 mL is needed for Ac, 5 mL for ammonia, and 6 mL for orthophosphate determination, then at least a 14 mL sample must be collected.

In a well-mixed system, liquid samples are only taken once (although the analysis may be conducted in replicate) because the sampling itself does not usually affect the quality of the sample (unless the samples are not filtered by omission).

- Also, the number of samples (and their volume) to be collected should be defined in accordance with the type of analysis, the volumes or masses required for the analytical determination of the particular parameters of interest and the number of replicates per analysis (e.g. 3 samples of 10 mL each for MLSS/MLVSS determination by triplicate, 20 mg TSS per every PHA analysis and 5 mg TSS for each glycogen analysis). In the case of samples focused on solids concentrations, there is deviation in the solids concentrations from sample to sample due to the nature of sampling procedures. Therefore, the sampling and analysis preferably need to be conducted in triplicate.
- Frequency of sample collection:
  a. If the maximum specific (initial) kinetic rates are to be determined (e.g. maximum specific acetate uptake rate or maximum orthophosphate uptake rate) then a higher number of samples must be taken at the beginning of the corresponding stage or phase (anaerobic, anoxic or aerobic). In particular, samples may need to be collected every 5 min during the first 30-40 min of duration of the batch activity test. The 5 min period is the minimum practical period needed for taking and handling a single (set of) sample(s).
  b. If only the stoichiometric ratios need to be assessed and not the kinetics (e.g. the anaerobic P-released/Ac-uptake ratio or anaerobic PHB formation/Ac uptake ratio), then the samples can be collected only at the beginning and end of each stage or phase to determine the total conversions of interest.
- To increase the data reliability and know the initial conditions of the sludge, it is strongly recommended to collect a series of sludge samples before any media is added. In particular for PHAs and glycogen, which require a long sampling time considering the fast conversion rates of the biomass, similar to MLSS and MLVSS analyses, samples are often collected in triplicate together with MLSS and MLVSS samples.
- Carefully define the maximum and minimum working volumes of the bioreactor:
  a. The estimation of the minimum final volume at the end of the test (after the collection of all the samples) will avoid problems with sampling (e.g. when the height of the final volume is lower than the height of the sampling port/tubing) and controlling the operational conditions (e.g. uncontrolled acid and base addition will occur if the tip of the pH electrode is not submerged leading to a pH shock, without noticing any problems in the reading of the pH meter). It can also prevent inadequate or insufficient aeration and mixing (extremely low volumes can lead to high oxygen intrusion, dead volumes, and biomass losses if splashed on the bioreactor walls).
  b. To estimate the minimum initial volume at the beginning of the test based on the minimum final volume and the volume required for sampling (taking into account the initial activated sludge volume and the addition of media and other solutions) and to verify that this volume does not exceed the maximum working volume of the bioreactor which will allow to avoid spillages and flooding. The potential increase in volume due to gas sparging (e.g. nitrogen or compressed air) must also be considered to define the maximum working volume.
- Once the number and frequency of the sample collections have been defined, then label all the plastic cups. Preferably, define a nomenclature and/or abbreviation that will allow you to easily identify and recognize the batch test, the sampling time and the parameter(s) of interest to be determined with that sample. Labelling both the plastic cups and the cover will help to easily identify the sample.
- A simple working sheet created in a spreadsheet can be rather useful to execute and keep track of the sampling collection and batch test execution. Furthermore, it can be used to keep a database of the different batch tests carried out. Table 2.2.10 contains an example of a working sheet.
- Organize all the required material within a relatively close radius of action around the batch setup so any delay in handling and preparing the samples can be avoided. Otherwise, it will be difficult to respect the initial 5 min frequency of sampling.

- If samples for PHA and glycogen determination are to be collected, carefully add 4-5 drops of the 37 % formaldehyde solution using a plastic Pasteur pipette. Add the formaldehyde solution inside a fume hood or at least in a well ventilated place. After addition, close the tubes immediately and keep them closed until the samples are added. Always wear plastic gloves and treat the used materials contaminated with formaldehyde as chemical residue in accordance with your local lab regulations.
- Calibrate all the meters (pH, DO and thermometer) less than 24 h prior to the execution of the tests and store the electrode/sensors in appropriate solutions until the execution of the tests, following the particular recommendations of the corresponding manufacturer or supplier and confirm that the readings are reliable.
- One should be aware that each sample taken needs immediate attention, handling and proper storage, before the next sample is taken (Table 2.2.2).

Table 2.2.2 Suggestions for the storage and preservation of samples as a function of the analytical determination of the parameter of interest.

EBPR-related parameter of interest	Material of sample container	Method of preservation	Maximum recommended time between sampling, preservation procedure and analysis
Total BOD	Plastic or glass	Cool to 1-5 °C; or, freeze to -20 °C and store in the dark.	24 h for samples stored at 1-5 °C; up to 1 month for frozen samples.
Soluble/dissolved BOD	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters and cool to 1-5 °C; or, freeze to -20 °C and store in the dark.	24 h for samples stored at 1-5 °C; up to 1 month for frozen samples.
Total COD	Plastic or glass	Cool to 1-5 °C; or, add concentrated H_2SO_4 to lower pH to 1-2, freeze to -20 °C and store in the dark .	24 h for samples stored at 1-5 °C; up to 6 months for acidified samples frozen and stored in the dark.
Soluble/dissolved COD	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters, cool to 1-5 °C.	24 h for samples stored at 1-5 °C; up to 6 months for acidified samples frozen and stored in the dark.
VFA	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters, cool to 1-5 °C; or, add concentrated H_2SO_4 to lower pH to 1-2, freeze to -20 °C and store in the dark.	24 h for samples stored at 1-5 °C; up to 6 months for acidified samples frozen and stored in the dark.
Total P	Plastic or glass acid washed (0.1 M HCl)	Add concentrated H_2SO_4 to lower pH to 1-2 and freeze to -20 °C.	6 months.
PO_4	Plastic or glass acid washed (0.1 M HCl)	Filter immediately after collection through 0.45 µm pore size filters and cool to 1-5 °C.	24 h.
NH_4	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters, cool to 1-5 °C; or, add concentrated H_2SO_4 to lower pH to 1-2, freeze to -20 °C and store in the dark.	24 h for samples stored at 1-5 °C; up to 21 days for acidified samples frozen and stored in the dark.
NO_2	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters, cool to 1-5 °C.	24 h.
NO_3	Plastic or glass	Filter immediately after collection through 0.45 µm pore size filters, cool to 1-5 °C; or, add concentrated HCl to lower pH to 1-2, freeze to -20 °C and store in the dark.	24 h for samples stored at 1-5 °C; 7 days for acidified samples frozen and stored in the dark.
PHAs	Plastic	After corresponding sampling and preparation procedure (see Section 2.2.2.5) store at -20 °C or -80 °C; freeze-dried samples can also be stored at -20 or -80 °C.	Up to 6 months.
Glycogen	Plastic or glass	After corresponding sampling and preparation procedure (see Section 2.2.2.5) store at -20 °C; after digestion also store at -20 °C.	Up to 6 months.
MLSS	Plastic or glass	Cool to 1- 5 °C.	2 days.
MLVSS	Plastic or glass	Cool to 1-5 °C.	24 h.

2.2.3.5 Activated sludge preparation

These procedures consider that batch activity tests can be performed as soon as possible after the collection of samples from full- or lab-scale systems or, in the worst-case scenario, within 24 h after collection. The execution of batch tests 24 h after the collection of activated sludge samples is not recommended due to potential changes that EBPR culture can experience during handling (unless the exposure time after collection is of particular interest for the execution of tests). Bearing in mind the previous comments, the following three procedures are recommended to prepare the activated sludge samples for the execution of batch activity tests:

- If batch activity tests can be executed in less than 1 h after collection of the sludge sample and if the sludge sample does not need to be washed:
  a. Adjust the temperature of the batch bioreactor where the tests will take place to the target temperature of the study.
  b. Collect the sludge:
     i. At the end of the aerobic tank or stage to carry out anaerobic batch tests.
     ii. At the end of the anaerobic stage or tank to perform anoxic or aerobic batch tests.
  c. Transfer the sludge sample to the bioreactor or fermenter where the batch activity tests will take place.
  d. Add the ATU solution (if applicable for the objective of the test) to a final concentration of 20 mg L^{-1} (see Section 2.2.3.3).
  e. Start a gentle mixing (50 - 100 rpm) and follow the temperature of the sludge sample by placing an external thermometer inside the bioreactor (if the setup does not have a built-in thermometer).
  f. Keep mixing until the sludge has reached the target temperature of the study.
  g. Keep the same redox conditions prevailing during collection until the batch activity tests are ready to start:
     i. Avoid the aeration of samples collected under anaerobic conditions. If available, use an airtight bioreactor and sparge nitrogen gas to avoid/reduce oxygen intrusion.
     ii. Avoid the aeration of samples collected under anoxic conditions and preferably add a nitrate solution to a final concentration of around 10 mg NO_3-N L^{-1} to preserve the anoxic environment while mixing.
     iii. Aerate the sludge samples collected in the aerobic tank or under aerobic conditions, keeping a dissolved oxygen concentration higher than 2 mg L^{-1}.
  h. Sludge for the execution of anaerobic batch tests: if nitrate is detected in the aerobic samples collected to perform these tests (see Section 2.2.2.3), the aeration must stop and the sludge sample should be gently mixed until nitrate is no longer observed. As soon as nitrate is no longer observed, the sludge can be immediately used to execute the anaerobic batch activity tests. Nitrate or nitrite detection strips (Sigma-Aldrich) can be used for a quick estimation of the presence of these compounds.
  i. Sludge to perform anoxic and aerobic batch activity tests can then be used to carry out the tests within 1 h of collection.
- If batch activity tests can be conducted in less than 1 h after collection of the sludge sample but the activated sludge sample needs to be washed:
  a. Adjust the temperature of the batch bioreactor where the tests will take place to the target temperature of the study. Collect the sludge at the end of the aerobic tank or stage.
  b. Wash the sludge in a mineral solution (see Section 2.2.3.3) as follows:
     i. Separate the biomass by settling or mild centrifugation (2,000-3,000 rpm for 5 min) and carefully remove the supernatant volume while avoiding losing the biomass.
     ii. Replace the supernatant volume with the same volume of nutrient solution and mix gently for 5 min.
     iii. Repeat the previous washing procedure at least one more time.
     iv. After the last washing cycle, separate the biomass by settling or mild centrifugation (2,000-3,000 rpm for 5 min) and re-suspend the sludge in the same volume of mineral solution previously added.
     v. The washed sample should have the same MLSS concentration as in the lab- or full-scale system where it was taken from. Thus, define and adjust the volume of the mineral solution added to re-suspend the washed sludge in order to reach the same MLSS concentration like in the original source.
  c. Transfer the washed sludge sample to the bioreactor or fermenter where the batch activity tests will take place.
  d. Add the ATU solution (if applicable for the objective of the tests) to a final concentration of 20 mg L^{-1} (see Section 2.2.3.3).

e. Start a gentle mixing (50-100 rpm) and follow the temperature of the sludge sample by placing an external thermometer inside the bioreactor (if the setup does not have a built-in thermometer).
f. Keep mixing until the sludge is exposed to the target temperature of the study for at least 30 min.
g. Start to aerate the sludge sample, keeping a dissolved oxygen concentration not lower than 2 mg L^{-1} while providing a gentle mixing until the batch activity test starts.
h. Due to the exposure of the sludge to aerated and non-aerated conditions, it is only recommended to use this sludge to execute batch activity tests that start with an anaerobic phase (anaerobic-(anoxic)-aerobic). The execution of batch activity tests that start with an anoxic or aerobic phase is not recommended because the washing steps may decrease the intracellular PHA contents and poly-P contents due to handling during the washing steps.

- If due to location and distance issues, the tests cannot be performed within less than 1 or 2 h after collection (but within 24 h):
  a. Adjust the temperature of the batch bioreactor to the target temperature of the study.
  b. Keep the sludge sample cold until the test is executed (e.g. by placing the bucket or jerry can in a fridge at 4 °C), avoiding aerating the sludge sample.
  c. Prior to the execution of the test, take the sludge sample out of the fridge, cool box or cold room.
  d. Mix the content gently in order to obtain a homogenous and representative sample with a similar MLSS concentration as in the original lab- or scale system where it was collected from.
  e. If the sample needs to be washed, wash the sludge in a mineral solution (see Section 2.2.3.3) as follows (otherwise, skip the washing step):
     i. Separate the biomass by settling or mild centrifugation (2,000-3,000 rpm for 5 min) and carefully remove the supernatant volume while avoiding losing the biomass.
     ii. Replace the supernatant volume with the same volume of nutrient solution and mix gently for 5 min.
     iii. Repeat the previous washing procedure at least one more time.
     iv. After the last washing cycle, separate the biomass by settling or mild centrifugation (2,000-3,000 rpm for 5 min) and re-suspend the sludge in the same volume of nutrient solution previously added.
     v. Since the washed sample should have the same MLSS concentration as in the lab- or full-scale system where it was taken from, define and adjust the volume of the mineral solution to obtain the same MLSS concentration as in the original source.
  f. Transfer the washed sludge to the bioreactor or fermenter where the batch activity test will take place.
  g. Add the ATU solution to a final concentration of 20 mg L^{-1} (see Section 2.2.3.3).
  h. Start to aerate the sludge sample, keeping the dissolved oxygen concentration higher than 2 mg L^{-1} while mixing gently.
  i. Follow the temperature of the sludge sample by placing an external thermometer inside the bioreactor (if the setup does not have a built-in thermometer).
  j. Keep aerating and mixing for at least 1 h (maximum 2 h) but ensure that the sludge is exposed to the target temperature of the study for at least 30 min.
  k. If nitrate is detected after the procedure to adjust the temperature, (see Section 2.2.2.3 on activated sludge sample preparation), the aeration must stop. Mix gently until nitrate is no longer observed. Afterwards, the sludge can be immediately used to start and execute an anaerobic phase test.
  l. If the sample was 'washed', it is only recommended to use this sludge to execute batch activity tests that start with an anaerobic phase (e.g. anaerobic-(anoxic)-aerobic), because the washing steps may decrease the intracellular PHA contents and poly-P contents due to handling during the washing steps. Thus, an anaerobic stage is always needed to replenish the PHA contents of the biomass.

2.2.4 Batch activity tests: execution

Once the experimental setup, materials, solutions, and activated sludge are ready, the corresponding batch activity test can be conducted. To facilitate the execution and for data track record and archiving purposes, an experimental implementation plan should be prepared in advance. Table 2.2.10 presents a template for an experimental implementation plan that can be used with necessary modifications for the execution of each of the batch activity tests described in the following sections. Due to the particular metabolism of EBPR cultures, EBPR batch activity tests can range from anaerobic to

anoxic and aerobic tests, including different combinations among them, depending on the purpose or goal of the test.

Thus, the following EBPR batch activity tests are presented in this chapter:

Test code no.	Redox conditions	Short description and purpose
EBPR.ANA.1	Anaerobic	Executed under the absence of an external carbon source to assess the endogenous anaerobic maintenance activity of EBPR cultures.
EBPR.ANA.2	Anaerobic	Performed after the addition of a defined concentration of carbon to determine the maximum anaerobic activity of EBPR cultures.
EBPR.ANA.3	Anaerobic	Carried out after the addition of a carbon source in excess to estimate the maximum activity of EBPR cultures under non-limiting carbon conditions.
EBPR.ANOX.1	Anoxic	Performed with activated sludge samples collected at the end of the anaerobic stage/phase.
EBPR.ANOX.2	Combined anaerobic-anoxic	Anaerobic-anoxic test conducted with sludge collected at the end of an aerobic stage.
EBPR.AER.1	Aerobic	Performed with sludge collected at the end of an anaerobic or anoxic stage to assess the aerobic EBPR activity.
EBPR.AER.2	Combined anaerobic-aerobic	Anaerobic-aerobic test conducted with sludge collected at the end of an aerobic stage.
EBPR.AER.3	Combined anaerobic-anoxic-aerobic in series	Anaerobic-anoxic-aerobic test carried out with sludge collected at the end of an aerobic stage to assess the sequential anaerobic, anoxic and aerobic EBPR activities.
EBPR.AER.4	Combined anaerobic-anoxic-aerobic in parallel	After the conduction of a common anaerobic phase, one anoxic and one aerobic test are performed in parallel with the same sludge to assess the anoxic and aerobic EBPR activities for comparison purposes.

2.2.4.1 Anaerobic EBPR batch activity tests

The length of an anaerobic batch test can last from 1 h to more than 8 h. Biomass is sensitive to pH and temperature, so the tests should be conducted at the temperature and pH of interest and fluctuations should be avoided. Tests should be executed under the absence of any electron acceptor (molecular oxygen, nitrate or nitrite) (e.g. truly anaerobic conditions). To avoid or minimize oxygen intrusion, it is recommended to use airtight reactors/fermenters and, if available, sparge N_2 gas continuously throughout the execution of the test. To remove nitrate present in the sample, ATU must be added during the sample preparation (before the sample is aerated) and the sludge can be gently mixed for a few minutes to remove any residual nitrate. Depending upon the purpose of the anaerobic batch activity test, the availability and presence of electron donors can vary. The following anaerobic tests are widely performed:

1. **Test EBPR.ANA.1** Performed under the absence of external carbon source to assess the endogenous anaerobic maintenance activity of EBPR cultures.
2. **Test EBPR.ANA.2** Carried out after the addition of a defined concentration of carbon (which should be fully consumed within the duration of the anaerobic test): to determine the maximum carbon uptake rate, maximum P-release rate, half-saturation constant for carbon uptake, and associated anaerobic stoichiometry such as P-released to carbon consumed ratio.
3. **Test EBPR.ANA.3** Executed after the addition of a carbon source in excess of the concentration that an EBPR culture could consume within the duration of the test: to estimate the maximum concentration of phosphorus that can be released and the maximum concentration of carbon that the EBPR cultures can consume under non-limiting carbon conditions.

Since the presence and type of carbon source play a major role in EBPR processes, sludge collection and preparation are of major importance. It is preferable to execute these tests with activated sludge collected at the end of the aerobic stage (to minimize the presence of an originally present carbon source) and/or after following a washing procedure. Thus, the following protocols for the execution of anaerobic batch tests are suggested depending upon the presence and availability of an external carbon source:

Test EBPR.ANA.1 Anaerobic batch EBPR tests performed under the absence of an electron donor

a. After sludge has been collected, prepared and transferred to the batch bioreactor (see Section 2.2.3.5), keep the sample aerated for at least 30 min while confirming that the pH and temperature are at the target value of interest. Otherwise, set up the corresponding set points (if automatic pH and temperature controllers are applied) or adjust manually. Wait until stable conditions are reached.
b. Once stable operating conditions are reached, around 20 min before the start of the test take the first samples of the water phase and biomass to determine the initial concentrations of the parameters of interest: C-source, total P, PO_4 and MLSS and MLVSS concentrations. Samples for the determination of PHAs and glycogen can also be collected to assess the anaerobic stoichiometric conversions. It is recommended to also take samples of the media to check and verify the initial concentrations.
c. For sampling, connect the syringe, open or release the lab clip or clamp that closes the sampling port, and pull and push the syringe several times until a homogenous sample is collected (usually around 5 times are required). Next, when the syringe is full, close the clip and remove the syringe.
d. Samples for the determination of soluble components must be immediately filtered (through 0.45 µm pore size filters). Other samples (e.g. PHAs, glycogen) need to be prepared in accordance with the corresponding protocols explained earlier in the chapter.
e. During the test execution, temporarily store the samples at 4 °C in the fridge or preferably in a cool box with ice.
f. 10 min before the start of the test stop the aeration and close the bioreactor.
g. If available, start to sparge N_2 gas and continue sparging until the end of the batch test. Provide an adequate outlet to allow the exit of N_2 gas and avoid building up overpressure (which can lead to flooding and biomass loss). If a continuous N_2 gas sparging throughout the batch test is not feasible, sparge N_2 gas for 10 min (alternatively another suitable gas could be used to remove the oxygen present and avoid its intrusion) and afterwards keep the bioreactor under airtight conditions.
h. Start the execution of the anaerobic test at ‘time zero’. Keep track of the execution time with the stopwatch.
i. Duration and sampling:
   i. If only the anaerobic endogenous maintenance P-release rate must be determined, the test can last for 1 h or maximum 2 h with continuous sampling every 15 min for the determination of PO_4 released by biomass (PAOs) under the absence of external carbon.
   ii. If the anaerobic endogenous maintenance glycogen conversion rate and stoichiometric conversions are also of interest, the anaerobic tests must be extended for up to 6 and 8 h (recommended). Samples for PO_4 determination can be collected every 30 min together with samples for PHA and glycogen determination.
   iii. Conclude the anaerobic test with the collection of samples for the determination of COD, Total P, PO_4 and MLSS and MLVSS concentrations, as well as for PHAs and glycogen (if applicable).
j. Ensure that considerable temperature and pH variations (higher than 1 °C or ± 0.1, for temperature and pH, respectively) do not take place during the execution of the batch test, and that DO readings remain below the detection limits. Make sure that all the electrodes used are recently calibrated before the execution of the test.
k. Organize the samples and ensure that all the samples are complete and properly labelled to avoid mixing of samples and other trivial mistakes.
l. Until the collected samples are analysed, preserve and store them as recommended by the corresponding analytical procedures.
m. Clean up the apparatus and take appropriate measures to keep and preserve the different sensors, equipment and materials.
n. Keep (part of) the sludge used in the test for possible further use (e.g. for microbial identification, see chapters 7 and 8).

Test EBPR.ANA.2 Anaerobic batch EBPR tests performed under a defined addition of an electron donor

a. Repeat steps ‘a’ to ‘g’ from Test EBPR.ANA.1.
b. For anaerobic tests performed with the presence of an external carbon source (electron donor), the tests start at 'time zero' with the addition of the real or synthetic wastewater (as a carbon source solution).
c. To execute the tests, the following MLVSS and RBCOD concentrations are suggested depending on the origin of the sludge samples:
   i. Sludge samples from full-scale activated sludge systems: add the RBCOD source to reach an initial RBCOD-to-MLVSS ratio in the bioreactor of between 0.025 and 0.050 mg COD mg VSS^{-1}.

For instance, mix the RBCOD source with the fresh activated sludge in such a way that the initial RBCOD concentration in the bioreactor is between 50 and 100 mg COD L^{-1} and the initial MLVSS concentration is around 2,000 mg VSS L^{-1}. A low RBCOD-to-MLVSS ratio is preferable to ensure the RBCOD consumption for the duration of the anaerobic test.

ii. Sludge samples from lab-scale activated sludge systems: the RBCOD source can be added to reach an initial RBCOD-to-MLVSS ratio in the bioreactor of between 0.05 and 0.10 mg COD mg VSS^{-1}. For example, the initial RBCOD and MLVSS concentrations after mixing can range between 100 and 300 mg COD L^{-1} and 2,000 and 3,000 mg VSS L^{-1}, respectively. Higher concentrations may also be acceptable as long as the COD is fully consumed within the length of the anaerobic stage. However, avoid COD concentrations higher than 800 mg COD L^{-1} because this may be inhibitory to biomass (author's personal observations).

d. After the addition of the wastewater, keep track of the execution and sampling times with a stopwatch.

e. Duration and sampling:

i. Tests can last between 2 and 4 h.

ii. To determine the anaerobic kinetic parameters, samples for the determination of soluble COD and PO_4 should be collected every 5 min in the first 30-40 min of execution of the test. After this period, the sampling frequency can be reduced to 10 or 15 min during the first 1 h, and later on to every 15 or 30 min until the test is finished.

iii. If the anaerobic kinetic conversion of PHAs and glycogen is of interest, samples should be collected at the same time as the samples for COD and PO_4 determination.

iv. For stochiometric conversions, samples for PHA and glycogen determination must be taken at the beginning and end of the test.

v. Conclude the anaerobic test with the collection of samples for the determination of C-source and/or COD (as desired depending upon the parameters of interest), total P, PO_4 and MLSS and MLVSS concentrations, as well as for PHAs and glycogen (if applicable).

f. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

Test EBPR.ANA.3 Anaerobic batch EBPR tests performed after the addition of an electron donor in excess

a. Repeat steps 'a' to 'g' from Test EBPR.ANA.1.

b. For anaerobic tests performed with the presence of an external carbon source (electron donor), the tests start at 'time zero' with the addition of the real or synthetic wastewater (as a carbon source solution).

c. To execute the tests, the following MLVSS and RBCOD concentrations are suggested depending on the origin of the sludge samples:

i. Sludge samples from full-scale activated sludge systems: Perform the anaerobic tests with an initial RBCOD-to-MLVSS ratio in the bioreactor higher than 0.15 mg COD mg VSS^{-1} after mixing the RBCOD source and the sludge. For instance, for sludge samples with MLVSS concentrations of around 2,000, add 300 mg COD L^{-1} of the RBCOD source of interest. Higher concentrations can be added but avoid adding more than 800 mg COD L^{-1} since this has been shown to be inhibitory for EBPR cultures (author's personal observation). If all RBCOD is consumed, more RBCOD can be added until it is not totally consumed. Monitoring the PO_4 concentration's profile during the execution of the test can be used as an indirect method to assess whether the sludge has reached its maximum RBCOD removal/uptake capacity. This can be applied if after an additional dose of RBCOD source there is not a considerable increase in PO_4 concentrations (e.g. less than 2-3 mg PO_4-P L^{-1} during 30-60 min, which corresponds to anaerobic endogenous P release).

ii. Sludge samples from lab-scale activated sludge systems: similarly to full-scale sludge samples, apply an initial RBCOD-to-MLVSS ratio in the bioreactor higher than 0.2 mg COD mg VSS^{-1} (after mixing the COD source and the sludge). Lab-scale cultures, particularly from EBPR systems, have a considerably high RBCOD removal capacity, which may require repeating the COD addition more than twice until no further COD uptake is observed. For instance, for sludge samples with a MLVSS concentration of between 2,000 and 3,000 mg L^{-1}, add at least 400 mg COD L^{-1} of the RBCOD source of interest, but avoid adding more than 800 mg COD L^{-1} (due to the potentially inhibitory effects on EBPR cultures). If all RBCOD is consumed, more RBCOD can be added until a residual COD is observed. Monitoring the PO_4 concentrations profile during

the execution of the test can be used as an indirect method to assess whether the sludge has reached its maximum RBCOD removal capacity. This approach can be applied if after an additional dose of RBCOD source there is not a considerable increase in PO_4 concentrations (e.g. less than 2-3 mg PO_4-P L^{-1} after 30-60 min).

d. Start to keep track of the execution time with a stopwatch just after the addition of the wastewater.
e. Duration and sampling:
   i. Tests can last more than 2-4 h for samples from full-scale systems and even longer for lab-scale systems depending on the poly-P and glycogen content of the biomass.
   ii. To determine the anaerobic kinetic parameters, samples for the determination of C-source and/or COD (depending upon the parameters of interest) and PO_4 must be collected every 5 min in the first 30 min of execution of the test. After this period, the sampling frequency can be reduced to every 10 or 15 min during the first 1 h, and later on to every 15 or 30 min until the test is finished.
   iii. If the anaerobic kinetic conversions of PHAs and glycogen are of interest, samples can be collected at the same time as the samples for C-source and/or COD and PO_4 determination.
   iv. For stochiometric conversions, samples for the determination of PHAs and glycogen must be taken at the beginning and end of the test.
   v. Prior to the end of the tests, add an additional concentration of COD. Wait for 10-15 min and take the last sample for COD and PO_4 determination. If no additional COD uptake and P release are observed, this will help to confirm whether the test was indeed performed under non-limiting COD conditions.
   vi. Conclude the anaerobic test with the collection of samples for the determination of C-source and/or COD, total P, PO_4 and MLSS and MLVSS concentrations, as well as for PHAs and glycogen (if applicable).
f. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

2.2.4.2 Anoxic EBPR batch tests

Anoxic EBPR batch tests are conducted to assess the simultaneous removal of orthophosphate and nitrate (or nitrite) by PAOs. They can be performed with activated sludge samples from full- or lab-scale systems, using real or synthetic wastewater/solutions. It is important that the EBPR biomass has enough intracellularly stored PHAs available when exposed to anoxic conditions as a carbon and energy source for P-uptake, glycogen formation, biomass growth and maintenance (Figure 2.2.1). In any case, no external carbon source should be added. Sludge samples can be collected at the end of the anaerobic stage or phase, as long as RBCOD has been fully consumed (and therefore absent from the activated sludge). To ensure the availability of PHAs, it is strongly advised to avoid washing the activated sludge or biomass in between the sludge collection and handling at the end of the anaerobic stage and the start of the anoxic batch test. Under exceptional circumstances (e.g. the suspected presence of toxic compounds), activated sludge samples may be washed as long as strictly anaerobic conditions can be created during the washing procedure (a complicated procedure in practice). Instead, the sludge can be collected at the end of the aerobic stage and the anoxic EBPR test can start with a preceding anaerobic batch test under the defined addition of an electron donor (similar to Test EBPR.ANA.2, described in Section 2.2.4.1). Thus, the following anoxic activity batch tests are suggested:

1. **Test EBPR.ANOX.1** Single anoxic EBPR test performed with activated sludge samples collected at the end of the anaerobic stage/phase.
2. **Test EBPR.ANOX.2** Combined anaerobic-anoxic EBPR batch test. The anoxic EBPR batch test is conducted after a preceding anaerobic phase with sludge collected at the end of the aerobic stage.

The description of each test is presented below:

Test EBPR.ANOX.1 Single anoxic EBPR batch tests

a. Collect the activated sludge sample in the full- or lab-scale plant at the end of the anaerobic stage. Prepare and transfer it to the batch bioreactor as described in Section 2.2.3.5. Keep the sample under anaerobic conditions for at least 30 min while confirming that the pH, DO and temperature are at the target values of interest (otherwise adjust and wait until stable conditions are reached).
b. Repeat steps 'b' to 'e' from Test EBPR.ANA.1.
c. If available, sparge N_2 gas continuously until the end of the batch test. Provide an adequate outlet to allow the exit of N_2 gas and avoid any overpressure. If continuous N_2 gas sparging throughout the batch test cannot be applied, sparge N_2 gas for 10 min (alternatively another

suitable gas can be used to remove the oxygen present and avoid its intrusion) and afterwards keep the bioreactor airtight.

d. Start the execution of the anoxic EBPR test at 'time zero' with the addition of nitrate or nitrite (depending upon the final electron acceptor of interest). The same operating conditions like those applied for the anaerobic tests must be applied to avoid the intrusion of oxygen (see Test EBPR.ANA.1).

e. Duration and sampling of the anoxic EBPR batch test:

i. The anoxic EBPR test can last between 2 and 4 h.

ii. Depending upon the final electron acceptor of interest:

- Anoxic EBPR tests performed with nitrate (NO_3^-) as the final electron acceptor:

  For activated sludge samples exposed to the presence of nitrate, up to 20 mg NO_3-N L^{-1} can be added at the beginning of the anoxic stage. On the other hand, it is not recommended to add more than 20 mg NO_3-N L^{-1} to activated sludge samples not regularly exposed to high nitrate concentrations. Nevertheless, if all the nitrate is consumed, an additional 20 mg NO_3-N L^{-1} can be added to extend the length of the anoxic stage until no further anoxic P-uptake is observed.

- Anoxic EBPR tests performed with nitrite (NO_2^-) as the final electron acceptor:

  Usually, activated sludge samples are not exposed to high nitrite concentrations, unless they are acclimatized to the presence of this electron acceptor (in exceptional/particular cases). Thus, for activated sludge samples exposed to the presence of nitrite, up to 20 mg NO_2-N L^{-1} can be added at the beginning of the anoxic stage. On the other hand, it is not recommended to add more than 10 mg NO_2-N L^{-1} to activated sludge samples not regularly exposed to high nitrite concentrations. In the latter case, if all the nitrite is consumed, an additional 10 mg NO_2-N L^{-1} can be added to ensure the availability of electron acceptor and the anoxic stage can be extended until no further anoxic P-uptake is observed.

iii. Since the analytical determination of nitrate or nitrite can not be determined as quickly as needed to monitor their presence during the test, nitrate and/or nitrite detection strips (Sigma-Aldrich) can be used to rapidly estimate their presence and to a certain degree of accuracy their concentration, and to assess whether the anoxic conditions are still present or if additional nitrate or nitrite must be dosed.

iv. For the estimation of the anoxic kinetic parameters, samples for the determination of C-source (or soluble COD depending upon the analytical parameter of interest) and PO_4 must be collected every 5 min in the first 30 min of execution of the test. After this period, the sampling frequency can be reduced to every 10 or 15 min during the first 1 h, and later on to every 15 or 30 min until the test is finished.

v. If the anoxic kinetic conversions of PHAs and glycogen are of interest, samples can be collected at the same time as the samples for PO_4 determination.

vi. To estimate the anoxic stochiometric conversions, samples for the analytical determination of total P, PO_4, NO_3 (or NO_2, and MLSS and MLVSS concentrations, as well as for PHAs and glycogen (if applicable), must be collected both at the start and at the end of the anoxic phase.

f. Conclude the anoxic test with the collection of the last samples needed for the estimation of the anoxic stoichiometric conversions.

g. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

Test EBPR.ANOX.2 Combined anaerobic-anoxic EBPR batch tests

a. Repeat steps 'a' to 'g' from Test EBPR.ANA.1 and steps 'b' to 'f' from Test EBPR.ANA.2. Afterwards, continue with the execution of the anoxic stage.

b. Immediately after the anaerobic stage is completed, the anoxic EBPR batch test can start immediately with the addition of nitrate or nitrite (Test EBPR.ANOX.1, step ‘d’). The same operating conditions like those applied for anaerobic tests (e.g. Test EBPR.ANA.1) must be applied to avoid the intrusion of oxygen.

c. Duration and sampling of the anoxic EBPR batch test: repeat step 'e' from Test EBPR.ANOX.1.

d. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

2.2.4.3 Aerobic EBPR batch tests

Aerobic EBPR batch tests can be executed to assess the orthophosphate uptake by PAOs in activated sludge

systems. They can be performed with activated sludge samples from full- or lab-scale systems, using real or synthetic wastewater/solutions. Similar to anoxic BPR tests, it is important that the EBPR biomass has enough intracellularly stored PHAs available when exposed to aerobic conditions as a carbon and energy source for P-uptake, glycogen formation, biomass growth and maintenance (Figure 2.2.1). To ensure the availability of intracellularly stored PHAs, aerobic batch tests must be (*i*) performed with activated sludge samples collected at the end of the anaerobic or anoxic stage/phase (depending upon the wastewater treatment plant configuration), or, (*ii*) carried out after an anaerobic batch test and before the aerobic test is conducted. In any case, no external carbon source should be added during the aerobic phase. Thus, samples can be collected at the end of the anaerobic stage or phase, as long as RBCOD is not present. Alternatively, the aerobic test can be performed in a sequential mode after an anaerobic-anoxic test (Test EBPR.ANOX.1) resulting in an anaerobic-anoxic-aerobic test or, in parallel to an anoxic test after the execution of an anaerobic test (Test EBPR.ANA.2) (Wachtmeister *et al.,* 1997). It is strongly recommended to avoid washing the activated sludge or biomass in between the sludge collection and handling from the anaerobic or anoxic stage and the start of the aerobic batch test to reduce the potential oxidation of the PHAs. If the biomass needs to be washed (e.g. due to the suspected presence of toxic compounds), then an anaerobic test should always be executed prior to the aerobic batch test under the defined presence of an electron donor (Test EBPR.ANA.2). Thus, the following aerobic EBPR batch tests (with and without preceding anaerobic or anoxic stages) can be proposed:

1. **Test EBPR.AER.1** A single aerobic EBPR test performed with sludge collected at the end of an anaerobic or anoxic stage to assess the aerobic EBPR activity on the intracellular polymers stored in the original source of sludge.
2. **Test EBPR.AER.2** Combined anaerobic-aerobic EBPR batch tests conducted to ensure a defined concentration of intracellular PHAs to cover the aerobic metabolic requirements using sludge collected at the end of an aerobic stage.
3. **Test EBPR.AER.3** Combined anaerobic-anoxic-aerobic EBPR batch tests in series carried out with sludge collected at the end of an aerobic stage to assess the sequential anaerobic, anoxic and aerobic EBPR activities after securing a defined concentration of intracellularly stored PHAs.
4. **Test EBPR.AER.4** Combined anaerobic-anoxic-aerobic EBPR batch tests in parallel performed with sludge collected at the end of an aerobic stage to assess the anoxic and aerobic EBPR activities in parallel after an anaerobic phase. Since the anaerobic test is common, the sludge has the same content of intracellularly stored polymers at the beginning of the anoxic and aerobic tests, so both anoxic and aerobic EBPR activities can be compared to each other and in some cases even conducted in parallel (usually the privilege of more experienced experimenters).

The description of the different steps involved in the execution of aerobic EBPR tests is as follows:

Test EBPR.AER.1 Single aerobic EBPR test

a. Collect the sludge in the anaerobic stage or anoxic stage following the recommendations for sampling and activated sludge preparation described in Section 2.2.3.5. It is important to notice that single aerobic tests can only be performed when the tests can be executed immediately after collection (and preferably avoiding a washing procedure).
b. After the activated sludge has been transferred, keep the same redox conditions as those prevailing in the collection tank (e.g. anaerobic or anoxic) as described in Section 2.2.3.5 for at least 30 min while confirming that the pH and temperature are at the target values of interest (otherwise adjust and wait until stable conditions are reached). Do not start to aerate the sample and, if available, sparge N_2 gas (or another gas available) to avoid oxygen intrusion.
c. Repeat steps 'b' to 'e' from Test EBPR.ANA.1.
d. The test starts at 'time zero' with the supply of air (or pure oxygen), ensuring that the DO concentration with regard to the DO saturation concentration at local conditions reaches at least 2.0 mg DO L^{-1} within the first 10 min of execution of the aerobic test and around 4-5 mg DO L^{-1} onwards.
e. After the air supply starts, keep track of the execution and sampling time with a stopwatch.
f. Duration and sampling of the aerobic stage:
   i. The aerobic EBPR test can last between 2 - 4 h.
   ii. The DO concentration in the bulk liquid can be monitored throughout the test with the use of a DO probe.
   iii. For the estimation of the aerobic kinetic parameters, samples for the determination of PO_4 must be collected every 5 min in the first 30-40 min of execution of the test. After this period, the sampling frequency can be reduced to every 10 or

15 min during the first 1 h, and later on to every 15 or 30 min until the test is finished.

iv. If the aerobic kinetic conversions of PHAs and glycogen are of interest, samples can be collected at the same time as the samples for PO_4 determination.

v. To estimate the aerobic stochiometric conversions, samples for the analytical determination of total P, PO_4, and MLSS and MLVSS concentrations, as well as for PHAs and glycogen (if applicable), must be collected both at the start and at the end of the aerobic phase. However, the total oxygen consumption must be determined by respirometry (as presented in Chapter 3).

g. Conclude the aerobic EBPR batch test with the collection of the last samples needed for the estimation of the aerobic stoichiometric conversions.
h. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

Test EBPR.AER.2 Combined anaerobic-aerobic EBPR batch tests

a. Execute the anaerobic test as follows:
   i. Repeat steps 'a' to 'g' from Test EBPR.ANA.1.
   ii. Afterwards, execute steps 'b' to 'e' from Test EBPR.ANA.2.
b. Immediately after the execution of the anaerobic test, the aerobic EBPR batch test can start with the sparging of compressed air or pure oxygen ensuring that the DO concentration with regard to the DO saturation concentration at local conditions reaches at least 2.0 mg DO L^{-1} within the first 10 min of execution of the aerobic test and around 4-5 mg DO L^{-1} afterwards.
c. Repeat step 'f' from Test EBPR.AER.1.
d. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

Test EBPR.AER.3 Combined anaerobic-anoxic-aerobic EBPR batch tests in series

a. Execute the anaerobic test as follows:
   i. Repeat steps 'a' to 'g' from Test EBPR.ANA.1.
   ii. Afterwards, execute steps 'b' to 'e' from Test EBPR.ANA.2.
b. Immediately continue with the execution of the anoxic test by repeating steps 'd' and 'e' from Test EBPR.ANOX.1.
c. After the anoxic test, continue with the execution of the aerobic stage by repeating steps 'd' to 'g' from Test EBPR.AER.1.
d. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

Test EBPR.AER.4 Combined anaerobic-anoxic-aerobic EBPR batch tests in parallel

a. This test requires two (preferably identical) reactors: one for the execution of an anaerobic-anoxic stage and another for the execution of a single aerobic test. This is achieved by transferring, at the end of the anaerobic test, a defined volume (usually 50 %) from the bioreactor where the anaerobic-anoxic test will take place to the second bioreactor where the aerobic test will be conducted. For this purpose, the steps described here below are recommended.
b. Execute the anaerobic test as follows:
   i. Repeat steps 'a' to 'g' from Test EBPR.ANA.1.
   ii. Afterwards, execute steps 'b' to 'e' from Test EBPR.ANA.2.
c. Once the anaerobic stage is completed, transfer 50 % of the activated sludge present in the anaerobic bioreactor to the empty bioreactor (aerobic bioreactor).
d. Continue with the execution of the anoxic test in the same bioreactor where the anaerobic test took place by repeating steps 'd' and 'e' from Test EBPR.ANOX.1.
e. In parallel, carry out the execution of the aerobic test in the second bioreactor (where sludge was transferred) by repeating steps 'd' to 'g' from Test EBPR.AER.1. Note that the execution of two (anoxic and aerobic) tests in parallel requires more advanced experimentation skills. Two alternative approaches are that either two persons execute the tests, or to carry out the tests one after another.
f. Repeat steps 'j' to 'n' from Test EBPR.ANA.1.

2.2.5 Data analysis

2.2.5.1 Estimation of stoichiometric parameters

Prior to estimation of the stoichiometric and kinetic parameters, a COD balance should be conducted to validate the results and confirm their quality and reliability (Barker and Dold, 1995). A rather important tool to assess the reliability of data is the COD balance. Theoretically, the COD must be conserved in a truly anaerobic system so that the total COD entering an anaerobic stage must be equal to the total COD that leaves the anaerobic stage (Wentzel *et al.*, 2008). Thus, assuming that soluble COD, intracellular PHAs and glycogen are the only carbon components involved in the chemical transformations, a COD balance can be performed as follows:

$$COD_{B,cons} + COD_{GLY,cons} = COD_{PHA,prod} \quad \text{Eq. 2.2.1}$$

Where:

$COD_{B,cons}$ is the concentration of biodegradable substrate, as COD, consumed during the duration of the anaerobic batch activity test, in mg COD L^{-1}.

$COD_{GLY,cons}$ is the concentration of intracellular glycogen consumed during the duration of the anaerobic batch activity test, in mg COD L^{-1}.

$COD_{PHA,prod}$ is the concentration of intracellular PHAs formed or stored during the duration of the anaerobic batch activity test, in mg COD L^{-1}.

Also, the percentage of error to close the COD balance (ΔCOD (%)) can be estimated as:

$$\% \text{ COD balance} = \left[1 - \frac{COD_{B,cons} + COD_{GLY,cons} - COD_{PHA,prod}}{COD_{B,cons} + COD_{GLY,cons} + COD_{PHA,prod}}\right] \cdot 100 \quad \text{Eq. 2.2.2}$$

Ideally, ΔCOD (%) should be lower than 1-5 % but values as high as 15 % are often reported and considered acceptable in view of, and depending on, data quality and the uncertainty that the determination of certain parameters creates (in particular glycogen). The reader is invited to consult Chapter 5 for more information on the assessment of data quality.

Similarly to the anaerobic transformations, the COD balance can be an important tool to assess the reliability of the data obtained in anoxic and aerobic batch activity tests (Ekama and Wentzel, 2008a,b). Thus, the total amount of final electron acceptors consumed should equal the total amount of electron donors oxidized. For the aerobic transformations that take place during an aerobic EBPR test, a COD balance can be performed as follows:

$$\Delta COD_{cons} = COD_{input} - COD_{output} = \Delta O_{2,cons} \quad \text{Eq. 2.2.3}$$

Assuming that PHAs, glycogen (GLY) and biomass (Bio) are the only COD components that change during the anoxic or aerobic phase, the net COD consumption can be calculated as follows:

$$COD_{PHA,cons} - COD_{GLY,prod} - COD_{Bio,prod} = \Delta O_{2,cons} \quad \text{Eq. 2.2.4}$$

Where:

$COD_{PHA,cons}$ is the total concentration of PHA consumption during the aerobic batch activity test, in mg COD L^{-1}.

$COD_{GLY,prod}$ is the total concentration of glycogen produced during the aerobic batch activity test, in mg COD L^{-1}.

$COD_{Bio,prod}$ is the total concentration of biomass produced during the duration of the aerobic batch activity test, in mg COD L^{-1}.

$\Delta O_{2,cons}$ is the total concentration of oxygen consumed in the aerobic batch activity test estimated based on respirometry and oxygen uptake rates (see Chapter 3 on Respirometry) in mg COD L^{-1}.

The percentage of error to close the COD balance (ΔCOD (%)) can be estimated as:

$$\% \text{ COD balance} = \left[1 - \frac{COD_{PHA,cons} - COD_{GLY,prod} - COD_{Bio,prod} - \Delta O_{2,cons}}{COD_{PHA,cons} + COD_{GLY,prod} + COD_{Bio,prod} + \Delta O_{2,cons}}\right] \cdot 100 \quad \text{Eq. 2.2.5}$$

Ideally, similar to the determination of COD balances for the anaerobic stage of EBPR batch activity tests, ΔCOD(%) should be lower than 1-5 % but values as high as 10 % can be considered acceptable. Chapter 5 provides different tools and approaches to assess the quality of the data obtained and execute the estimation of the COD balances with a smaller uncertainty about their reliability.

COD balances can also be determined and applied to anoxic EBPR activity tests where, for instance, nitrate or nitrite act as the final electron acceptor. When applying a COD balance on EBPR batch activity tests executed with a different final electron acceptor rather than oxygen, the approach would be similar, but: (*i*) the equivalent COD concentrations of the final electron acceptor should be determined and expressed in COD units based on its capacity to accept electrons, and (*ii*) the corresponding maximum stoichiometric yields (Y) of the metabolic conversions on the final electron acceptor of interest (for instance, nitrate or nitrite) should be known. For tests conducted with nitrate as the final electron acceptor, the maximum stoichiometric yields estimated by Kuba *et al.* (1996) can be used. Last but not least, the total consumption of the final electron acceptor during the execution of the anoxic batch EBPR test should be estimated based on the experimental methods described in Chapter 3.

Once the quality and reliability of the data have been confirmed and validated, the stoichiometric and kinetic parameters can be computed. Often, Net P-released, glycogen conversion, and PHAs produced per organic carbon or COD consumed ($Y_{VFA\_PO4,An}$, $Y_{Gly/VFA,An}$, $Y_{VFA\_PHA,An}$, $Y_{VFA\_PHB,An}$ and $Y_{VFA\_PHV,An}$ ratios, respectively) are the stoichiometric parameters of interest to assess the anaerobic stoichiometry of the EBPR processes (Table 2.2.1).

Regarding the anaerobic stoichiometric parameters, often the most common carbon source used for the execution of EBPR batch activity tests is acetate (Ac). Figure 2.2.5 shows a graphic representation of the determination of both the stoichiometry and kinetic parameters for Ac consumed and orthophosphate released in an anaerobic batch activity test. It should be noted that the Net $P_{released}$ due to Ac uptake should be estimated after excluding the secondary PO_4 release ($r_{PP\_PO4,Sec,An}$). However, because the secondary PO_4 release occurs continuously throughout the anaerobic test as a consequence of the anaerobic maintenance requirements of the cells (though usually it can only be observed after the carbon source is depleted), the accumulated PO^4 released due to anaerobic maintenance should be excluded from the total PO_4 release observed.

Thus, the Net $P_{released}$ can be estimated as follows:

$$\text{Net } P_{released} = [(\text{Total } PO_4\text{-}P_{released})] - [(r_{PP\_PO4,Sec,An}) \cdot (\text{test duration})] \quad \text{Eq. 2.2.6}$$

Where:

Net $P_{released}$ is the PO_4 released due to Ac uptake only, mg PO_4-P L^{-1}.

$r_{PP\_PO4,Sec,An}$ corresponds to the PO_4 release rate due to anaerobic maintenance requirements of the biomass, mg PO_4-P L^{-1} h^{-1}.

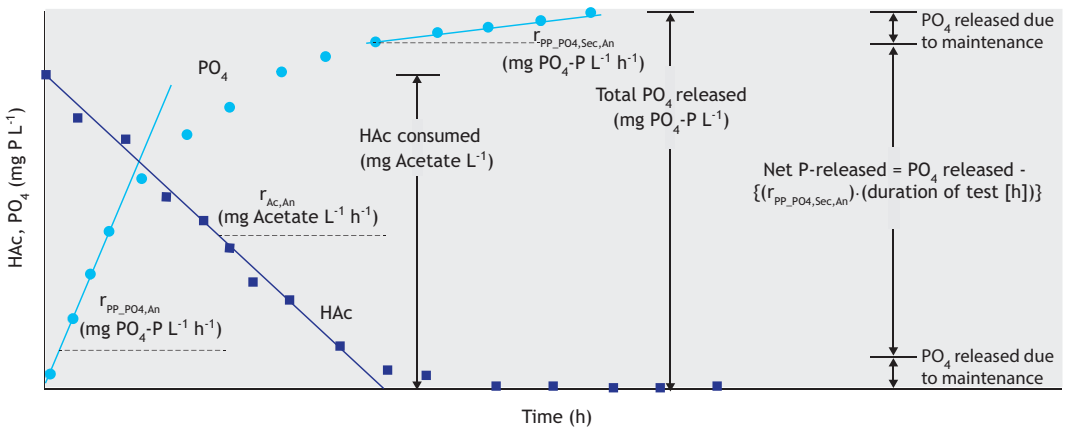

Figure 2.2.5 Example of the determination of the maximum anaerobic volumetric kinetic rates for acetate consumption (Ac) and orthophosphate released (PO_4) in an anaerobic batch activity test where all the carbon is consumed. For the estimation of the Net P released, the secondary P release ($r_{PP\_PO4,Sec,An}$)(corresponding to the anaerobic endogenous maintenance requirements which occur throughout the anaerobic test) must be excluded (Net P released = [(Total PO_4 released)] - [($r_{PP\_PO4,Sec,An}$)·(duration of the test)]). The Net P released should be used for the determination of the anaerobic PO_4-to-Ac stoichiometric ratio ($Y_{Ac\_PO4,An}$).

The net P released should be used for the determination of the anaerobic PO_4/Ac stoichiometric ratio ($Y_{Ac\_PO4,An}$). Thus, for instance, if in a batch activity test the carbon is fully consumed, the anaerobic net P-released/Ac-consumed ratio (which can also be referred to as the P/C ratio) of a given culture can be calculated as follows:

$$Y_{Ac_{PO4},An} = \frac{\text{Net } P_{released}}{S_{Ac,cons}} = \frac{S_{PO_4,ini} - S_{PO_4,final}}{S_{Ac,ini} - S_{Ac,final}} \quad \text{Eq. 2.2.7}$$

Where:

$S_{Ac,cons}$ is the concentration of acetate consumed in the batch activity test, mg L^{-1}.

$S_{PO4,ini}$ is the orthophosphate concentration in the bulk liquid at the beginning of the batch activity test, mg PO_4-P L^{-1}.

$S_{PO4-P,final}$ is the orthophosphate concentration in the bulk liquid at the time when the acetate concentration is consumed or at the end of the anaerobic batch activity tests if not all the acetate is depleted, mg PO_4-P L^{-1}.

$S_{Ac,ini}$ is the concentration of acetate in the bulk liquid at the beginning of the batch activity test, mg L^{-1}.

$S_{Ac,final}$ is the concentration of acetate in the bulk liquid at the end of the batch activity test, mg L^{-1}.

However, if the carbon is not depleted (e.g. when carbon is added in excess or the duration of the test is relatively too short to allow the full consumption of the carbon source), then the difference between the initial and final concentration of the compounds can be divided by the difference between the initial and final concentrations of carbon (net carbon consumed). A similar approach can be applied for the determination of other anaerobic stoichiometric ratios of interest (like the anaerobic glycogen hydrolysis and PHAs produced per C-source consumed ratios). Often, different units are used to report the stoichiometry of the anaerobic conversions, like the use of C-mol or P-mol instead of mg Ac or mg PO_4-P in scientific publications. For this purpose, Appendix I contains a series of coefficients for the conversion of units of certain compounds of interest (e.g. to convert the units of orthophosphate from g PO_4-P to P-mmol). Unlike the anaerobic stoichiometric parameters, the anoxic and aerobic stoichiometric parameters cannot be determined in a similar straightforward manner. Because EBPR cultures have different intracellular metabolic conversions occurring simultaneously under anoxic or aerobic conditions (poly-P uptake, glycogen replenishment, growth and maintenance) (Table 2.2.1), the net consumption of electron donors (PHAs) and final electron acceptors (oxygen, nitrate or nitrite) is the combined result of these four overlapping metabolic activities (Smolders *et al.,* 1994b).

Nevertheless, Smolders *et al.* (1994b) and Kuba *et al.* (1993, 1996) proved that using metabolic modelling, the four aerobic or anoxic metabolic processes are dependent on the ratio between the ATP produced per NADH consumed during aerobic or anoxic respiration, the so called 'δ-ratio' or 'δ-value' (Smolders *et al.,* 1994b; Kuba *et al.,* 1996). This means that for EBPR cultures, the aerobic or anoxic value of δ can be determined to consequently estimate the values of the different aerobic or anoxic stochiometric parameters, respectively. Furthermore, despite different δ values having been reported in literature for diverse microbial populations (ranging from 1.3 to 2.2) (Lopez-Vazquez *et al.,* 2009a), for EBPR cultures, the effect of the δ-value is rather insensitive to the aerobic and anoxic stoichiometric ratios within this range of δ values. The latter suggests that the aerobic and anoxic stoichiometric parameters are also insensitive and there may not be any need for their determination if the plant or lab-scale system operates under regular operating and environmental conditions. However, if the determination of the δ value is needed, two tests can be performed with and without the presence of orthophosphate in the bulk liquid and by measuring the oxygen uptake rate (OUR) by respirometry (Chapter 3). By computing the differences in P-uptake rates and OUR between the tests performed with and without the presence of orthophosphate in the bulk liquid, the δ value can be estimated as described by Smolders *et al.* (1994b). Alternatively, mathematical modelling can be applied to estimate the δ value based on the anoxic and/or aerobic profiles of PHAs, glycogen, orthophosphate, growth rate and maintenance requirements (Lopez-Vazquez *et al.,* 2009a). Should there be interest in the determination of δ for EBPR cultures, the reader may need to refer to cited references for further reading since this procedure falls out of the scope of this book.

As a reference and guide, Table 2.2.3 shows different parameters of interest for lab-scale EBPR cultures enriched under different operating conditions (e.g. carbon source) and dominated by either PAOs or GAOs.

Table 2.2.3 Typical stoichiometric parameters of interest for lab-scale enriched EBPR cultures cultivated under standard conditions (20 °C, pH 7, 7-8 d SRT).

	Stoichiometric parameter	Common notation	Units	Typical values	Reference
	Lab-scale PAO culture enriched with acetate				
ANAEROBIC	Anaerobic orthophosphate release to acetate uptake ratio	$Y_{Ac\_PO4,An}$	P-mol C-mol^{-1}	0.50	Smolders *et al.* (1994a)
	Anaerobic glycogen utilization to acetate uptake ratio	$Y_{Gly/Ac,An}$	C-mol C-mol^{-1}	0.50	
	Anaerobic PHA formation to acetate uptake ratio	$Y_{Ac\_PHA,An}$	C-mol C-mol^{-1}	1.22	
	Anaerobic PHB formation to acetate uptake ratio	$Y_{Ac\_PHB,An}$	C-mol C-mol^{-1}	1.10	
	Anaerobic PHV formation to acetate uptake ratio	$Y_{Ac\_PHV,An}$	C-mol C-mol^{-1}	0.12	
	Anaerobic PH_2MV formation to acetate uptake ratio	$Y_{Ac\_PH2MV,An}$	C-mol C-mol^{-1}	N/A	

AEROBIC	Aerobic poly-P formation to PHA consumption ratio	$Y_{PHA\_PP,Ox}$	P-mol C-mol^{-1}	3.68	Smolders *et al.* (1994b)
	Aerobic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ox}$	C-mol C-mol^{-1}	0.90	
	Aerobic PAO biomass growth to PHA consumption ratio	$Y_{PHA\_PAO,Ox}$	C-mol C-mol^{-1}	0.74	
	Aerobic maintenance rate of PAO	$m_{PAO,Ox}$	C-mol C-mol^{-1} h^{-1}	4x10^{-3}	
	Aerobic poly-P formation to oxygen consumption ratio	Y_{PP}	P-mol C-mol^{-1}	3.27	
	Aerobic glycogen formation to oxygen consumption ratio of PAO	$Y_{Gly,PAO}$	C-mol mol-O_2^{-1}	3.92	
	Aerobic PAO biomass growth to oxygen consumption ratio	Y_{PAO}	C-mol mol-O_2^{-1}	2.44	
	Aerobic endogenous respiration rate of PAO	$m_{PAO,O2}$	mol-O_2 C-mol^{-1} h^{-1}	4.5x10^{-3}	
	Lab-scale PAO culture enriched with propionate				
ANAEROBIC	Anaerobic orthophosphate release to propionate uptake ratio	$Y_{Pr\_PO4,An}$	P-mol C-mol^{-1}	0.42	Oehmen *et al.* (2005c)
	Anaerobic glycogen utilization to propionate uptake ratio	$Y_{Pr\_Gly,An}$	C-mol C-mol^{-1}	0.32	
	Anaerobic PHA formation to propionate uptake ratio	$Y_{Pr\_PHA,An}$	C-mol C-mol^{-1}	1.23	
	Anaerobic PHB formation to propionate uptake ratio	$Y_{Pr\_PHB,An}$	C-mol C-mol^{-1}	0.04	
	Anaerobic PHV formation to propionate uptake ratio	$Y_{Pr\_PHV,An}$	C-mol C-mol^{-1}	0.55	
	Anaerobic PH_2MV formation to propionate uptake ratio	$Y_{Pr\_PH2MV,An}$	C-mol C-mol^{-1}	0.65	
AEROBIC	Aerobic poly-P formation to PHA consumption ratio	$Y_{PHA\_PP,Ox}$	P-mol C-mol^{-1}	3.34	Oehmen *et al.* (2007)
	Aerobic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ox}$	C-mol C-mol^{-1}	1.06	
	Aerobic PAO biomass growth to PHA consumption ratio	$Y_{PHA\_PAO,Ox}$	C-mol C-mol^{-1}	0.80	
	Aerobic maintenance rate of PAO	$m_{PAO,Ox}$	C-mol C-mol^{-1} h^{-1}	4x10^{-3}	
	Aerobic Poly-P formation to oxygen consumption ratio	Y_{PP}	P-mol C-mol^{-1}	3.34	
	Aerobic Glycogen formation to oxygen consumption ratio of PAO	$Y_{Gly,PAO}$	C-mol mol-O_2^{-1}	6.16	
	Aerobic Biomass growth to oxygen consumption ratio	Y_{PAO}	C-mol mol-O_2^{-1}	2.03	
	Aerobic endogenous respiration rate of PAO	$m_{PAO,O2}$	mol-O_2 C-mol^{-1} h^{-1}	4.5x10^{-3}	
	Lab-scale DPAO culture enriched with acetate				
ANAEROBIC	Anaerobic orthophosphate release to acetate uptake ratio	$Y_{Ac\_PO4,An}$	P-mol C-mol^{-1}	0.50	Smolders *et al.* (1994a),
	Anaerobic glycogen utilization to acetate uptake ratio	$Y_{Gly/Ac,An}$	C-mol C-mol^{-1}	0.50	Kuba *et al.* (1996)
	Anaerobic PHA formation to acetate uptake ratio	$Y_{Ac\_PHA,An}$	C-mol C-mol^{-1}	1.22	
	Anaerobic PHB formation to acetate uptake ratio	$Y_{Ac\_PHB,An}$	C-mol C-mol^{-1}	1.10	
	Anaerobic PHV formation to acetate uptake ratio	$Y_{Ac\_PHV,An}$	C-mol C-mol^{-1}	0.12	
	Anaerobic PH_2MV formation to acetate uptake ratio	$Y_{Ac\_PH2MV,An}$	C-mol C-mol^{-1}	N/A	
ANOXIC	Anoxic poly-P formation to PHA consumption ratio	$Y_{PHA\_PP,Ax}$	P-mol C-mol^{-1}	0.46	Kuba *et al.* (1996)
	Anoxic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ax}$	C-mol C-mol^{-1}	1.27	
	Anoxic PAO biomass growth to PHA consumption ratio	$Y_{PHA\_PAO,Ax}$	C-mol C-mol^{-1}	1.63	
	Anoxic maintenance rate of PAO	$m_{PAO,Ax}$	C-mol C-mol^{-1} h^{-1}	3.64x10^{-3}	
	Anoxic poly-P formation to NO_3 consumption ratio	$Y_{NO3\_PP,Ax}$	P-mol N-mol^{-1}	0.414	
	Anoxic glycogen formation to NO_3 consumption ratio	$Y_{NO3\_Gly,Ax}$	C-mol N-mol^{-1}	0.35	
	Anoxic PAO biomass growth to NO_3 consumption ratio	$Y_{NO3\_PAO,Ax}$	C-mol N-mol^{-1}	0.57	
	Anoxic endogenous respiration rate of PAO on NO_3	$m_{PAO,NO3}$	N-mol C-mol^{-1} h^{-1}	3.27x10^{-3}	
	Lab-scale GAO culture enriched with acetate				
ANAEROBIC	Anaerobic glycogen utilization to acetate uptake ratio	$Y_{Gly/Ac,An}$	C-mol C-mol^{-1}	1.12	Zeng *et al.* (2003a)
	Anaerobic PHA formation to acetate uptake ratio	$Y_{Ac\_PHA,An}$	C-mol C-mol^{-1}	1.86	
	Anaerobic PHB formation to acetate uptake ratio	$Y_{Ac\_PHB,An}$	C-mol C-mol^{-1}	1.36	
	Anaerobic PHV formation to acetate uptake ratio	$Y_{Ac\_PHV,An}$	C-mol C-mol^{-1}	0.46	
	Anaerobic PH_2MV formation to acetate uptake ratio	$Y_{Ac\_PH2MV,An}$	C-mol C-mol^{-1}	0.04	
AEROBIC	Aerobic glycogen formation to PHA consumption ratio	$Y_{PHA\_Gly,Ox}$	C-mol C-mol^{-1}	0.95	Zeng *et al.* (2003a)
	Aerobic GAO biomass growth to PHA consumption ratio	$Y_{PHA\_GAO,Ox}$	C-mol C-mol^{-1}	0.75	
	Aerobic maintenance rate of GAO	$m_{GAO,Ox}$	C-mol C-mol^{-1} h^{-1}	3.06x10^{-3}	
	Aerobic glycogen formation to oxygen consumption ratio	Y_{Gly}	C-mol mol-O_2^{-1}	4.89	
	Aerobic PHA degradation to oxygen consumption ratio of GAO	$Y_{PHA,GAO}$	C-mol mol-O_2^{-1}	2.18	
	Aerobic endogenous respiration rate of GAO	$m_{GAO,O2}$	mol-O_2 C-mol^{-1} h^{-1}	3.51x10^{-3}	

2.2.5.2 Estimation of kinetic parameters

Regarding the anaerobic kinetics, the most important parameters are the maximum specific consumption rate of carbon source or VFA ($q_{VFA,An}$), the maximum specific P-release rate ($q_{PP\_PO4,An}$), PHA formation rate ($q_{VFA\_PHA,An}$) and the endogenous ATP maintenance coefficient ($m_{ATP,An}$) (Table 2.2.1). They can be computed by plotting the experimental data (y-axis) versus time (x-axis) and fitting the experimental data obtained in the anaerobic batch activity tests using linear regression. Because one is interested in the maximum rates, a linear regression approach can be applied by fitting the very first set or group of experimental data points obtained at the beginning of the batch activity test. This is the main reason why the sampling frequency within the first 30-40 min of execution of the batch activity tests is set to 5 min. Preferably the linear regression must be carried out by plotting the concentrations and fitting more than 4-5 experimental data points by linear regression while achieving a statistical determination coefficient (R^2) not lower than 0.90-0.95. With the estimation of the linear regression equation (of the form: 'y = Ax + B'), the maximum volumetric kinetic rates of the parameters of interest can be determined with the 'A' coefficient of the linear regression expression which corresponds to the 'slope' of the set of data points. This will result in the determination of the maximum volumetric rates (usually reported in units such as mg L^{-1} h^{-1} or g m^{-3} d^{-1}). Figure 2.2.5 illustrates the estimation of the maximum anaerobic kinetic rates for an EBPR culture. The reader is referred to Chapter 5 for a more detailed description of other alternative and advanced statistical methods and tools for the determination of the rates. For activated sludge samples from full-scale systems, the rates can be expressed as maximum specific kinetic rates by dividing the volumetric rates (or values of the slopes) by the concentration of activated sludge volatile suspended solids (VSS). However, due to the particular dynamic behaviour of the intracellular compounds present in EBPR cultures, often the maximum specific kinetic rates are expressed in terms of the active biomass fraction (excluding the presence of intracellular compounds) quantified according to the following approximate expression:

Active biomass fraction = MLVSS – PHA – glycogen

Eq. 2.2.8

From a microbiological perspective, the previous equation is not an accurate expression due to the potential accumulation of non-biodegradable organics and, logically, the intrinsic metabolic activity of every single cell. Nevertheless, it is a commonly accepted estimate for experimental purposes in the wastewater treatment practice. Similarly to carbon and phosphate compounds (particularly for lab-cultivated cultures), the active biomass fraction can also be expressed in C-mol units instead of mg VSS. For this purpose the elemental composition of the biomass is applied (see Appendix I with the corresponding conversion unit factors for different compounds). Commonly, once the maximum specific kinetic rates are found they can be reported for the compound of interest as mg g VSS^{-1} h^{-1} or mg g VSS^{-1} d^{-1}. Similarly, the anaerobic endogenous ATP maintenance coefficient ($m_{ATP,An}$) can be determined based on the profile of orthophosphate concentration recorded during the execution of an anaerobic EBPR batch activity test performed under the absence of any carbon source (Section 2.2.4.1). This maximum volumetric rate resembles or corresponds to the endogenous P-release rate ($m_{PP\_PO4,Sec,An}$) observed in anaerobic tests once the carbon source is depleted (Fig. 2.2.6). Then, $m_{ATP,An}$ is equivalent to $m_{PP\_PO4,Sec,An}$ (Wentzel *et al.,* 1989a; Smolders *et al.,* 1994a). A similar linear regression approach can be used for the determination of the maximum specific kinetic rates for the aerobic and anoxic batch activity tests (tables 2.2.2 and 2.2.3). Usually, orthophosphate uptake, biomass growth, PHA degradation, glycogen formation and aerobic maintenance requirements are the kinetic parameters of interest dependent on the final electron acceptor available. Figure 2.2.6 displays an example to illustrate the determination of the maximum kinetic rates in an aerobic (or anoxic) batch activity test.

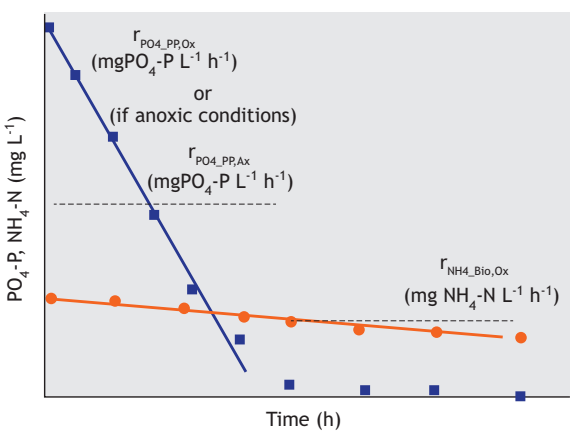

Figure 2.2.6 Example of the determination of the maximum aerobic (or anoxic) volumetric kinetic rates for orthophosphate uptake (PO_4) and ammonia consumption (NH_4) in an aerobic (or anoxic) batch test.

It is important to mention that the maximum specific biomass growth rate ($q_{PHA\_Bio,Ox}$) cannot be computed straightforwardly by following the increase in biomass concentrations during the cycle. Instead, the maximum specific ammonia (NH_4) consumption rate ($q_{NH4\_Bio,Ox}$) divided by the nitrogen composition of the active biomass fraction (0.20 N-mol per C-mol biomass) (Smolders *et al.,* 1995; Zeng *et al.,* 2003a; Lopez-Vazquez *et al.,* 2007; Welles *et al.,* 2014) can be used. However, the latter approach may be valid as long as nitrification is absent, no chemical precipitation or adsorption of NH_4 occurs and the relatively small differences allow a satisfactory determination of NH_4. Often, differences in NH_4 concentrations may be negligible or fall into the standard error of the analytical technique. This may complicate the determination process.

The experiments presented in this chapter focus on the relative conversions of intracellular and soluble compounds present in the water phase. It does not cover or tackle the consumption profiles nor the analysis of final electron acceptors (such as oxygen consumption, oxygen uptake rates, or nitrate uptake rates). These parameters are presented and discussed in Chapter 3 dealing with respirometry.

The determination of the aerobic maintenance coefficient of EBPR cultures is of major importance. For the determination of this parameter, extended aeration tests (regularly of at least 24 h) must be executed under the absence of an external carbon source. After 24 h, the aerobic ATP requirements ($m_{ATP,Ox}$) can be determined based on the oxygen consumption or uptake rate (OUR) (as O_2 mol consumed per mol active biomass per hour) and the aerobic maintenance requirements be computed with the following expression as a function of δ (with an average typical value of around 1.75-1.80 for enriched EBPR cultures):

$$m_{O2} = \frac{1.125\, m_{ATP,Ox}}{2.25\delta + 0.5} \qquad \text{Eq. 2.2.9}$$

2.2.6 Data discussion and interpretation

The results from the EBPR batch activity tests will provide important information regarding not only the biomass activity under different operating and environmental conditions but also about the general state of the biomass and some directions regarding the dominant microbial populations present in the sludge sample. Concerning the latter, the reader is invited to consult chapters 7 and 8. Furthermore, the identification of the dominant microbial species is an important complement to a better understanding of the EBPR process activities.

2.2.6.1 Anaerobic batch activity tests

As PAOs are the only organisms known to release phosphate during the anaerobic uptake of VFA, the anaerobic P-released/C-uptake or P-released/COD ratios, $Y_{C\_PO4,An}$ such as $Y_{VFA\_PO4,An}$, $Y_{Ac\_PO4,An}$ or $Y_{Pr\_PO4,An}$, may be considered one of the most suitable indicators to assess the PAO and GAO activity of EBPR cultures. A high P-released/C-uptake ratio has been considered as an indicator for PAO presence, whereas a low ratio has been considered to indicate the significant presence of GAOs. Contradictorily, such a consideration has been the subject of controversy (Schuler and Jenkins, 2003; Oehmen *et al.,* 2007; Lopez-Vazquez *et al.,* 2008b; Welles *et al.,* 2015b). Such a controversy arises due to the broad range of ratios reported in literature, ranging from values as low as 0.025 to even 0.75 P-mol C-mol^{-1} or higher (Schuler and Jenkins, 2003). Several studies with highly enriched PAO cultures revealed that factors other than the presence of GAOs affect the anaerobic P released/C uptake as well. For instance, the carbon source, the pH and the poly-P content of the PAOs and the specific clades of PAOs enriched in the study have been shown to affect the P-released/C-uptake ratio (Smolders *et al.,* 1994a; Filipe *et al.,* 2001a; Zhou *et al.,* 2008; Acevedo *et al.,* 2012). Consequently, it is not advisable to use only this ratio as the direct and sole indicator to assess the EBPR activity of an activated sludge. Nevertheless, it may well be used to provide a rough estimation of the dominant metabolisms prevailing in the activated sludge. If supported by molecular techniques (chapters 7 and 8 from this book), it can offer an adequate and more complete overview of the biomass activity. Thus, based on observations drawn from past research, different P/C ratios performed under standard conditions (20 °C, pH 7.0) and fed with acetate may indicate (Schuler and Jenkins, 2003):

P/C ratio (P-mol C-mol^{-1})	Dominant metabolism
< 0.25 →	GAO-dominated metabolism
0.25-0.50 →	Intermediate PAO-GAO metabolism
> 0.50 →	PAO-dominated metabolism

Figure 2.2.7 shows different anaerobic P-released/C-uptake ratios reported in literature as a function of the amount of phosphorus accumulated in the sludge on a TSS concentration basis expressed as the P/TSS ratio of

the sludge. Interestingly, the phosphorus content of the sludge (in terms of mg P g VSS^{-1}) has been shown to have a strong correlation with the anaerobic P-released/C-uptake ratio. As observed in Figure 2.2.7, an anaerobic P/C ratio higher than 0.50 is usually observed when the P/TSS content of the enriched PAO sludge is higher than 0.10 g P g TSS^{-1}. Thus, probably an activated sludge system will have a satisfactory EBPR activity when the anaerobic P-released/C-uptake and sludge P/TSS ratios are around or higher than 0.50 P-mol C-mol^{-1} and 0.10 mg P mg TSS^{-1}, respectively. Lower values could suggest that the EBPR activity of the system may be limited by certain operational or environmental factors such as (*i*) the relatively abundant presence of GAOs instead of PAOs, (*ii*) intrusion of electron acceptors into the anaerobic phase (like oxygen, NO_3, NO_2), (*iii*) the addition of Al of Fe salts for chemical P-removal, or occasionally (*iv*) the presence of inhibitory or toxic compounds. Under such circumstances the activated sludge system must be carefully revised to implement the corresponding corrective measures.

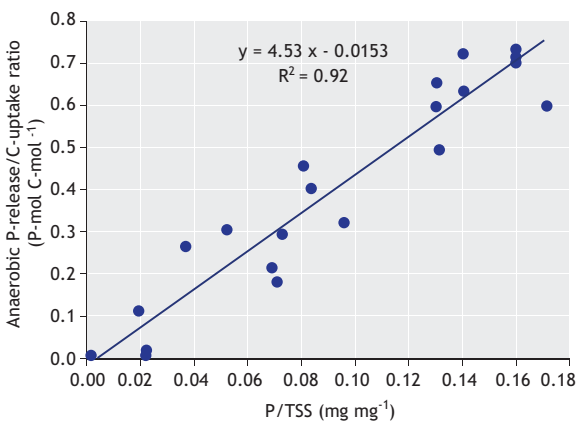

Figure 2.2.7 The anaerobic P-released/C-uptake ratio as a function of the P/TSS ratio of the biomass as reported in literature (adapted from Schuler and Jenkins, 2003; and Welles *et al.,* 2015b).

Under certain circumstances, the EBPR activity tests may be conducted under non-standard conditions (pH different to 7.0) using real wastewater or other carbon sources rather than acetate. For comparison and benchmarking purposes, the P/C ratio may be corrected using the expressions developed by Smolders *et al.* (1994a) or Filipe *et al.* (2001a) respectively, for acetate-fed cultures:

$$Y_{Ac\_PO4,An} = 0.19 \cdot pH - 0.85 \qquad \text{Eq. 2.2.10}$$

$$Y_{Ac\_PO4,An} = 0.16 \cdot pH - 0.55 \qquad \text{Eq. 2.2.11}$$

Also, the use of real wastewater or other C sources rather than acetate (like propionate, butyrate or glucose) will lead to a lower anaerobic P/C ratio than those reported for EBPR cultures where the PAO metabolism (or also PAOs) is dominant. Such lower P/C ratios can be, besides a low P/TSS content as previously discussed, a consequence of the lower energy needed for PHA storage or due to a higher involvement of GAO metabolism (possibly caused by the presence of GAOs). If the glycogen consumption/C-uptake ratio does not increase and remains within the commonly reported ratio for PAO-dominated systems of 0.35-0.50 C-mol C-mol^{-1} (Smolders *et al.,* 1994b; Schuler and Jenkins, 2003), the system can be assumed to be robust and stable, particularly if the intracellular P/TSS ratio is not considerably lower than 0.10 in enriched cultures. However, if under such circumstances the glycogen consumption/C-uptake ratio, $Y_{GLY/Ac,An}$, increases above 0.35-0.50 C-mol C-mol^{-1}, then the GAO metabolism (or the presence of GAOs themselves) may start to dominate the system and ultimately lead to the process deterioration as long as this ratio continues to increase (ultimately being able to reach a $Y_{GLY/Ac,An}$ of 1.12 C-mol C-mol^{-1}) in combination with a decrease in the P/TSS content below 0.10 mg P mg TSS^{-1}.

In parallel, PHA synthesis/C-uptake ratios higher than 1.33 C-mol C-mol^{-1} will be observed as a consequence of a higher synthesis of PHV and PH_2MV. PHV/C-uptake ratios higher than 0.10 and up to 0.25-0.30 may be observed together with the formation of PH_2MV. These values probably indicate the potential deterioration of the EBPR activity and efficiency. Regarding the anaerobic kinetic rates, in particular the initial maximum anaerobic uptake rate of carbon sources, different values have been reported for PAO and GAO lab-scale dominated cultures (mostly sequencing batch reactors, SBR) and full-scale systems such as modified UCT (University of Cape Town), Phoredox (which stands for phosphorus reduction oxidation), and PhoStrip (from phosphorus stripping) (Wentzel *et al.*, 2008) (Table 2.2.4).

Table 2.2.4 Maximum initial specific anaerobic C-source uptake rates reported in literature for lab-scale and full-scale EBPR systems.

Lab-scale EBPR systems		
Dominant microorganism/metabolism and system	$q_{VFA,An}$ in C-mol C-mol^{-1} h^{-1}	Reference
PAOs - SBR	0.27	Smolders *et al.* (1994a)
	0.20	Filipe *et al.* (2001b)
	0.20	Kuba *et al.* (1996)
	0.20	Brdjanovic *et al.* (1997)
	0.20	Lopez-Vazquez *et al.* (2007)
GAOs - SBR	0.24	Filipe *et al.* (2001a)
	0.16-0.18	Zeng *et al.* (2003a,b)
	0.20	Lopez-Vazquez *et al.* (2007)
	0.19	Lopez-Vazquez *et al.* (2009a)
Full-scale EBPR systems		
Dominant microorganism/metabolism	$q_{VFA,An}$ in mg Ac g VSS^{-1} h^{-1}	Reference
PAOs - Modified UCT	22	Lopez-Vazquez *et al.* (2008a)
	19	Lopez-Vazquez *et al.* (2008a)
	47	Kuba *et al.* (1997a,b)
	7-31	Kuba *et al.* (1997b)
PAOs - Phoredox	14	Lopez-Vazquez *et al.* (2008a)
	21	Lopez-Vazquez *et al.* (2008a)
	11	Lopez-Vazquez *et al.* (2008a)
	14	Lopez-Vazquez *et al.* (2008a)
PAOs - Sidestream PhoStrip	9	Lopez-Vazquez *et al.* (2008a)
	23	Brdjanovic *et al.* (2000)

Similarly to the P-released/C-uptake ratio, the kinetic rates seem to be dependent on the poly-P content of the sludge (Schuler and Jenkins 2003, Welles *et al.,* 2016, submitted), ranging from 0.02 to 0.20 C-mol C-mol^{-1} h^{-1}. However, in most studies conducted with medium poly-P contents and P/Ac ratios around 0.5 P-mol C-mol^{-1}, the observed uptake rates converge around 0.20 C-mol C-mol^{-1} h^{-1}.

Similarly, rates observed in the tests performed with activated sludge samples from full-scale EBPR systems lie in between 17 and 22 mg Ac g VSS^{-1} h^{-1} (Lopez-Vazquez *et al.,* 2008a).

Temperature plays a major role in the different EBPR microbial processes (Brdjanovic *et al.,* 1997, 1998c, Table 2.2.5).

Table 2.2.5 Arrhenius temperature coefficients (θ) reported in literature to describe the maximum specific kinetic rates of the different EBPR metabolic processes occurring in lab-scale and full-scale EBPR systems (Meijer, 2004).

Parameter	θ^*	Reference
$q_{VFA,An}$	1.094 ($e^{0.090}$)	Brdjanovic *et al.* (1998c); Meijer (2004)
$m_{ATP,An}$	1.071 ($e^{0.069}$)	Smolders *et al.* (1995); Murnleitner *et al.* (1997)
$q_{PHA,Ox}$	1.129 ($e^{0.121}$)	Brdjanovic *et al.* (1998); Meijer (2004)
$q_{PHA\_Gly,Ox}$	1.125 ($e^{0.118}$)	Meijer (2004)
$q_{PO4\_PP,Ox}$	1.031 ($e^{0.031}$)	Murnleitner *et al.* (1997); Brdjanovic *et al.* (1998c)
$q_{PAO,Ox}$	1.081 ($e^{0.078}$)	Brdjanovic *et al.* (1997)
$m_{ATP,Ax}$	1.071 ($e^{0.090}$)	Murnleitner *et al.* (1997)
$m_{ATP,Ox}$	1.071 ($e^{0.090}$)	Murnleitner *et al.* (1997)

* The number in brackets displays the value of the Arrhenius temperature coefficient (θ) in terms of the Euler number.

2.2.6.2 Aerobic batch activity tests

For comparison purposes, the observed maximum kinetic rates must be standardized using Arrhenius coefficients. Based on observations from Brdjanovic *et al.* (1997, 1998) and other authors (Smolders *et al.*, 1995, and Murnleitner *et al.*, 1997), Meijer (2004), through the development of the TUDelft model and the execution of several modelling studies, defined suitable Arrhenius temperature coefficients to best fit the maximum kinetic rates of EBPR cultures for different operating lab- and full-scale systems.

Although the aerobic kinetic rates reported in literature for lab-scale EBPR systems are consistent (Table 2.2.6), the values observed in full-scale EBPR systems may vary widely (from modified UCT systems to BIODENIPHO -biological denitrification nitrification and phosphorus removal- plants) (Table 2.2.7).

Table 2.2.6 Maximum aerobic kinetic rates reported in literature for enriched EBPR cultures.

Culture or system	$q_{PO4\_PP,Ox}$ P-mol C-mol^{-1} h^{-1}	$q_{PHA\_Gly,Ox}$ C-mol C-mol^{-1} h^{-1}	$q_{PAO,Ox}$ C-mol C-mol^{-1} h^{-1}	$m_{ATP,Ox}$ mol ATP C-mol^{-1} h^{-1}	Reference
SBR	0.055	0.080	0.014-0.016	$1.9x10^{-3}$	Smolders *et al.* (1994b)
SBR	0.046	-	0.13	$1.2x10^{-3}$	Brdjanovic *et al.* (1997)
SBR	0.083	-	-	$1.7x10^{-3}$	Welles *et al.* (2014)

Table 2.2.7 Maximum initial specific aerobic P-uptake rates reported in literature for full-scale EBPR systems.

Dominant microorganism/metabolism	$q_{PO4\_PP,Ox}$ mg P g VSS^{-1} h^{-1}	Reference
PAOs - Modified UCT	19.2	Lopez-Vazquez *et al.* (2008a)
	9.0	Lopez-Vazquez *et al.* (2008a)
	13	Kuba *et al.* (1997a, b)
	4-6	Kuba *et al.* (1997b)
PAOs - Phoredox	8.0	Lopez-Vazquez *et al.* (2008a)
	9.1	Lopez-Vazquez *et al.* (2008a)
	6.2	Lopez-Vazquez *et al.* (2008a)
	6.3	Lopez-Vazquez *et al.* (2008a)
PAOs - Sidestream PhoStrip	9.8	Lopez-Vazquez *et al.* (2008a)
	2.2	Brdjanovic *et al.* (2000)
PAOs - Pilot-scale BIODENIPHO	4	Meinhold *et al.* (1999)

Such considerable differences are logical since the lab-scale systems are highly enriched with PAOs (based on similar studies PAOs can probably compose more than 80-90 % of the total populations) with little variability in the percentage of enrichment (Lopez-Vazquez *et al.,* 2009a; Welles *et al.,* 2014, 2015a), whereas in full-scale systems PAOs may comprise between 3 and 20 % of the total active biomass fraction (Lopez-Vazquez *et al.,* 2008a). Moreover, in full-scale systems performing biological nitrogen and phosphorus removal, EBPR cultures are exposed to alternating anaerobic-anoxic-aerobic (A^2O) stages that may reduce the availability of intracellular PHAs after the sequential exposure to anoxic and aerobic conditions (since both anoxic and aerobic metabolic activities require PHAs as the carbon and energy source). Based on the data provided in Table 2.2.7, it can be seen that values higher than 10 mg P g VSS^{-1} h^{-1} are not common. Such kinetic rates can be considered rather fast since, as previously discussed, full-scale systems often tend to have aerobic hydraulic retention times (HRT) of several hours (e.g. of at least 6-8 h and longer) which will favour the uptake of orthophosphate from the bulk liquid. Aerobic P-uptake rates ($q_{PO4\_PP,Ox}$) lower than 10 mg P g VSS^{-1} h^{-1} will not be nsatisfactory depending upon the length of the aerobic stage (though extended aeration periods must be avoided)

(Brdjanovic *et al.,* 1998c). Another important aspect to consider is whether the plant is prone to chemical P-precipitation because its occurrence will favour the chemical P- removal to the detriment of the EBPR process.

2.2.6.3 Anoxic batch activity tests

The simultaneous removal of orthophosphate and nitrate (or nitrite) is highly desirable due to the potential savings in energy and operational costs, while at the same time keeping or maintaining a P-removal activity that can be comparable to that observed under anaerobic-aerobic conditions (Kuba *et al.,* 1996). However, to a certain extent, it has been a matter of controversy particularly due to the rather variable and inconsistent anoxic P-removal activities observed in full-scale systems (Hu *et al.,* 2002) and theoretically decreased P-removal potentially caused by lower PAO biomass yields. Table 2.2.8 shows an overview of different anoxic P-removal activities observed in full-scale systems (anaerobic-anoxic (A^2) and anaerobic-anoxic-oxic (A^2O) plants) that are compared to the aerobic P-removal activities of the same systems (following the protocols presented in this chapter as introduced by Murnleitner *et al.*, 1997).

Table 2.2.8 Maximum initial anoxic kinetic rates reported in literature for lab-enriched denitrifying EBPR cultures.

Culture or system	$q_{PO4\_PP,Ax}$	$q_{PHA\_Gly,Ax}$	$q_{PAO,Ax}$	m_{Ax}	Reference
	P-mol C-mol^{-1} h^{-1}	C-mol C-mol^{-1} h^{-1}	C-mol C-mol^{-1} h^{-1}	C-mol C-mol^{-1} h^{-1}	
SBR, PAOs, A^2 system	0.1	0.8	0.05	3.6x10^{-3}	Kuba *et al.* (1996)
SBR, PAOs, A^2 system	0.02-0.63[a]	0.0025	-	-	Carvalho *et al.* (2007)
SBR, PAOs, A^2 system	0.58[a]	0.9[a]	-	-	Zeng *et al.* (2003b)
SBR, PAOs, A^2O system	0.33[a]	-	-	-	Saito *et al.* (2004)

[a] Units: P-mmol g VSS^{-1} h^{-1}

Table 2.2.9 Maximum initial specific anoxic P-uptake rate and its relationship with maximum aerobic P-uptake rate reported for full-scale EBPR systems.

Dominant microorganism/metabolism	$q_{PO4\_PP,Ox}$ mg P g VSS^{-1} h^{-1}	$q_{PO4\_PP,Ax}$ mg P g VSS^{-1} h^{-1}	$q_{PO4\_PP,Ax}$ / $q_{PO4\_PP,Ox}$ %	Reference
PAOs - Modified UCT	19.2	5.9	31 %	Lopez-Vazquez *et al.* (2008a)
	9.0	2.1	23 %	Lopez-Vazquez *et al.* (2008a)
	13	6	46 %	Kuba *et al.* (1997a,b)
	4-6	1.2-1.6	20-40 %	Kuba *et al.* (1997b)
PAOs - Phoredox	8.0	1.9	23 %	Lopez-Vazquez *et al.* (2008a)
	9.1	4.4	48 %	Lopez-Vazquez *et al.* (2008a)
	6.2	0.6	9 %	Lopez-Vazquez *et al.* (2008a)
	6.3	0.0	0 %	Lopez-Vazquez *et al.* (2008a)
PAOs - Sidestream PhoStrip	9.8	3.3	34 %	Lopez-Vazquez *et al.* (2008a)
	2.2	1.7	80 %	Brdjanovic *et al.* (2000)
PAOs - Pilot-scale BIODENIPHO	4	2	54 %	Meinhold *et al.* (1999)

As observed in Table 2.2.9, the anoxic P-uptake rate scarcely reaches more than 5 mg PO_4-P g VSS^{-1} h^{-1} and, even under certain circumstances, it is very low or absent. In any case, the different anoxic P activities are a combined reflection of (*i*) the level of enrichment of denitrifying PAOs capable of using oxygen and nitrate (and/or of other EBPR cultures and side-populations involved in the denitrification process) (Kerr-Jespersen and Henze, 1993; Meinhold *et al.,* 1999; Saad *et al.,* 2016, submitted), and/or (*ii*) a measure of the level of denitrifying capacity induced in PAOs (Kuba *et al.,* 1996, 1997; Wachtmeister *et al.,* 1997). In any case, the level of exposure of the activated sludge system will favour the growth of denitrifying EBPR populations and their induction. Thus, higher anoxic P-uptake activities can be expected in plant configurations operated with defined pre-denitrification stage.

2.2.7 Example

2.2.7.1 Description

To illustrate the execution of an EBPR batch activity test, data from an anaerobic-aerobic test (Test EBPR.AER.2) performed at 10 °C with a lab-enriched EBPR culture is presented in this section. Test EBPR.AER.2 was carried out to determine the anaerobic stoichiometry and the anaerobic and aerobic kinetics of the EBPR processes. Thus, the batch activity test was performed in a 2.5 L bioreactor. All the equipment, apparatus and materials were prepared as described in Section 2.2.3. pH and DO sensors were calibrated less than 24 h before the test execution. The test lasted 4.5 h and was composed of a 2.25 h anaerobic stage (created by continuously sparging N_2 gas throughout the test) followed by 2.25 h aerobic stage (created by supplying compressed air in excess, reaching a DO concentration higher than 4 mg L^{-1}). Prior to the batch test, 1.25 L of concentrated EBPR sludge collected at the end of the aerobic phase of a lab-scale bioreactor was transferred to the bioreactor and acclimatized for 30 min at 10 °C under slow mixing (100 rpm) at pH 7.0 following the recommendations described in Section 2.2.3.5. Activated sludge preparation for tests was performed in less than 1 h after sludge collection. Afterwards, 20 min before the start of the test, samples for the determination of the parameters of interest were collected (in accordance with the execution of Test EBPR.AER.2).

The test started with the addition of 1.25 L synthetic media containing 350 mg COD L^{-1} as Ac (other macro- and micro-nutrients as well as 20 mg L^{-1} ATU were included in synthetic media in accordance with Section 2.2.3.3). Because the test was executed at 10 °C, the temperature of the synthetic media was adjusted to 10 °C in a water bath operated at the same temperature before addition. Because the main objective was to determine the anaerobic stoichiometry and the anaerobic and aerobic kinetics of the EBPR processes, samples were collected more frequently (every 5 min) in the first 30 min of each anaerobic and aerobic phase. Immediately after collection, all the samples were prepared, preserved and stored prior to the analytical determination of the parameters of interest (e.g. PO_4, MLSS, MLVSS, and PHAs, among other parameters) as described in Section 2.2.3.4 “Material preparation”. All the collected samples were analysed as described in Section 2.2.2.5. In particular, the intracellularly stored PHAs were determined following the protocol for the determination of PHB and PHV, and glycogen by the acid-hydrolysis and extraction methods (Smolders *et al.,* 1994a). Other PHA compounds, like PH_2MV, were not measured because acetate was the carbon source supplied and therefore it was expected that PHB and PHV would comprise most of the PHAs. Table 2.2.10 shows the experimental implementation plan of the execution of the test.

2.2.7.2 Data analysis

Following up on the results from the experiment shown in Table 2.2.10, Figure 2.2.8 shows the results from the test displayed in the implementation plan and also an estimation of the maximum volumetric kinetic rates by applying linear regression. The different anaerobic and aerobic conversions of the parameters of interest are shown in Table 2.2.11, while Table 2.2.12 displays an estimation of the different anaerobic and aerobic stoichiometric and kinetic parameters of interest.

Overall, the results of the batch activity test (Figure 2.2.8) show the typical phenotype of a PAO-dominated sludge: full Ac uptake in the anaerobic stage coupled with anaerobic P release, PHA production and glycogen consumption, while full PO_4 uptake was observed in the aerobic phase together with PHA utilization, glycogen formation and slight NH_4 consumption. Furthermore, the relatively low VSS/TSS ratio observed at the beginning and end of the test (of around 0.72-0.73) is typical for EBPR systems due to poly-P accumulation (which is reflected in a higher ash content) when compared to systems that perform organic matter removal only (with VSS/TSS ratios usually not lower than 0.80) (Wentzel *et al.,* 2008).

Table 2.2.10 Example of an experimental implementation plan for the execution of a batch activity test (Type Test No. EBPR.AER.2) performed with a lab-enriched EBPR sludge at 10 °C using synthetic influent at pH 7.0.

Combined anaerobic-aerobic EBPR batch tests — **Code: EBPR.AER.2**

Date:	Thursday 17.12.2015 9:00 h	**Experimental procedure in short:**	**Time (h:min)**
Description:	Tests at 10 °C, pH 7, artificial substrate and enriched PAO culture	1. Confirm availability of sampling material and required equipment	08:00
Test No.:	3 of 6	2. Confirm calibration and functionality of the system, meters and sensors	08:10
Duration	4,5 h (270 min)	3. Transfer 1.25 L sludge to the batch reactor	08:20
Substrate:	Synthetic: Acetate (350 mg L^{-1}) + minerals	4. Keep aerobic conditions with gentle mixing and air sparging at T and pH set	08:40
Sampling point:	Middle mixed liquor height in the SBR	5. 20 min before starting, take sample for initial conditions (EBPR.AER.2(3.1))	08:40
Samples No.:	EBPR.AER.2(1-22)	6. Stop aeration and start sparging by N_2 gas	08:50
Total sample volume:	305 mL	7. Start cycle, add 1.25 L of sysnthetic media (0 min)	09:00
	(10 mL for MLVSS, 12 mL for PHA, 4.5 mL for Glycogen,	8. Continue sampling program according to schedule (5 min)	09:05
	6 mL for other samples)	9. Stop sparging with N_2 gas, start addition of air (135 min)	11:15
Reactor volume:	2.5 L	10. Stop sampling and aeration (after 270 min)	13:30
		11. Organize the samples and clean the experimental equipment and space	13:45
		12. Ensure all equipment is switched off and samples are handled properly	14:00

Sampling schedule

Time (min)	-20	0	5	10	15	20	25	30	40	50	60	90	135
Time (h)	-0.33	0.00	0.08	0.17	0.25	0.33	0.42	0.50	0.67	0.83	1.00	1.50	2.25
Sample No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Parameter	ANAEROBIC PHASE												
HAc (C-mmol L^{-1})	5.83[1]		4.85	4.57	3.98	3.48	2.87	2.21	1.35	0.43	0	0	0
PO_4-P (P-mmol L^{-1})	0[1]		0.24	0.45	1.01	1.35	1.69	2.14	2.85	3.01	3.04	3.07	3.11
NH_4-N (N-mmol L^{-1})	1.32[1]			1.34								1.26	1.39
PHA (C-mmol)	12.27											20.04	20.08
Glycogen (C-mmol)	15.09											12.68	12.71
MLSS and MLVSS (mg L^{-1})	See table												See table

[1] Average value of the concentration present in the synthetic substrate and and liquid phase of the sludge sample prior the start of the test

Sampling schedule (continued)

Time (min)	140	145	150	155	160	165	180	195	215	270
Time (h)	2.33	2.42	2.50	2.58	2.67	2.75	3.00	3.25	3.58	4.50
Sample No.	13	14	15	16	17	18	19	20	21	22
Parameter	AEROBIC PHASE									
HAc (C-mmol L^{-1})										
PO_4-P (P-mmol L^{-1})	3.00	2.73	2.45	2.20	1.90	1.64	0.84	0	0	0
NH_4-N (N-mmol L^{-1})				1.11			1.09	1.07	1.06	1.05
PHA (C-mmol)							15.55			11.72
Glycogen (C-mmol)							15.04			15.90
MLSS and MLVSS (mg L^{-1})										See table

MLSS & MLVSS measurements

Sampling point	Cup No.	W1	W2	W3	W2-W1	W2-W3	MLSS	MLVSS	Ratio
Start anaerobic phase[2]	1	0.08835	0.16525	0.10792	0.07690	0.05733	7,690	5,733	0.75
	2	0.08835	0.16553	0.10997	0.07718	0.05556	7,718	5,556	0.72
	3	0.08834	0.16435	0.10903	0.07601	0.05532	7,601	5,532	0.73
						Average	7,670	5,607	0.73
End anaerobic/Start aerobic phase	4	0.08858	0.12437	0.09606	0.03579	0.02831	3,579	2,831	0.79
	5	0.08848	0.12564	0.09646	0.03716	0.02918	3,716	2,918	0.79
	6	0.08914	0.12527	0.09648	0.03613	0.02879	3,613	2,879	0.80
						Average	3,636	2,876	0.79
End aerobic phase	7	0.08868	0.12859	0.09952	0.03991	0.02907	3,991	2,907	0.73
	8	0.08764	0.12716	0.09881	0.03952	0.02835	3,952	2,835	0.72
	9	0.08722	0.12622	0.09800	0.03900	0.02822	3,900	2,822	0.72
						Average	3,948	2,855	0.72

[2] Sample taken before substrate addition

Biomass composition

Sampling point	Start Aner.	End Aner.	End Aer.
MLSS (mg L^{-1})	3,835	3,636	3,948
MLVSS (mg L^{-1})	2,804	2,876	2,855
Ratio	0.73	0.79	0.72
Ash (mg L^{-1})	1,031	760	1,093
PHB (mg L^{-1})	241.7	392.0	232.1
PHV (mg L^{-1})	20.9	37.3	18.6
PHA (mg L^{-1})	262.6	429.3	250.8
Glycogen (mg L^{-1})	423.7	343.3	429.2
%(PHA+Gly) $MLVSS^{-1}$	32.0	37.0	31.0
Active biomass (mg L^{-1})	2,117	2,103	2,175
Active biomass (Cmmol L^{-1})	81.4	80.9	83.6

Note:

Acetate (CH_2O)	30.03	mg C-$mmol^{-1}$
Ortho-Phosphate (PO_4-P)	31.00	mg P-$mmol^{-1}$
Ammonium (NH_4-N)	14.00	mg N-$mmol^{-1}$
PHB ($CH_{1.5}O_{0.5}$)	21.52	mg C-$mmol^{-1}$
PHV ($CH_{1.6}O_{0.4}$)	20.02	mg C-$mmol^{-1}$
Glycogen ($CH_{10/6}O_{5/6}$)	27.00	mg C-$mmol^{-1}$
Biomass ($CH_{2.09}O_{0.54}N_{0.20}$)	26.00	mg C-$mmol^{-1}$

Table 2.2.11 Summary of the anaerobic and aerobic conversions observed in the example of the batch activity test (Type Test No. EBPR.AER.2) performed with a lab-enriched EBPR sludge at 10 °C using synthetic influent at pH 7.0.

Parameter	Unit	Anaerobic phase			Aerobic phase		
		Start [Time: 0]	End [Time: 135 min]	Anaerobic conversion	Start [Time: 135 min]	End [Time: 270 min]	Aerobic conversion
Ac	C-mmol L^{-1}	5.20	0.00	-5.20	0.00	0.00	0.00
PO_4-P	P-mmol L^{-1}	0.00	3.11	3.11	3.11	0.00	-3.11
NH_4-N	N-mmol L^{-1}	1.32	1.39	0.07	1.39	1.05	-0.34
PHB	C-mmol L^{-1}	11.23	18.21	6.99	18.21	10.79	-7.43
PHV	C-mmol L^{-1}	1.05	1.86	0.82	1.86	0.93	-0.93
PHAs (PHB+PHV)	C-mmol L^{-1}	12.27	20.08	7.80	20.08	11.72	-8.36
Glycogen	C-mmol L^{-1}	15.69	12.71	-2.98	12.71	15.90	3.18

Table 2.2.12 Summary of the anaerobic and aerobic stoichiometric and kinetic parameters observed in the example of the batch activity test (Type Test No. EBPR.AER.2) performed with a lab-enriched EBPR sludge at 10 °C using synthetic influent at pH 7.0.

Conversion	Symbol	Unit	Estimated value
Anaerobic stoichiometry			
Net P-released to Ac uptake ratio[a]	$Y_{Ac\_PO4,An}$	P-mol C-mol^{-1}	0.57
PHA production to Ac uptake ratio	$Y_{Ac\_PHA,An}$	C-mol C-mol^{-1}	1.50
PHV production to PHB production ratio	$Y_{PHV/PHB,An}$	C-mol C-mol^{-1}	0.12
Glycogen consumption to Ac uptake ratio	$Y_{Gly/Ac,An}$	C-mol C-mol^{-1}	0.57
Anaerobic kinetic rates[b]			
Maximum volumetric Ac uptake rate	$r_{Ac,An}$	C-mmol L^{-1} h^{-1}	6.15
Maximum specific Ac uptake rate	$q_{Ac,An}$	C-mol C-mol^{-1} h^{-1}	0.075
Maximum volumetric PO_4 release rate	$r_{PP\_PO4,An}$	P-mmol L^{-1} h^{-1}	4.42
Maximum specific PO_4 release rate	$q_{PP\_PO4,An}$	P-mmol L^{-1} h^{-1}	0.054
Secondary anaerobic PO_4 release rate	$r_{PP\_PO4,Sec,An}$	P-mmol L^{-1} h^{-1}	0.063
Anaerobic maintenance coefficient	$m_{PP\_PO4,An}$	P-mol C-mol^{-1} h^{-1}	7.69 E-04
Aerobic kinetic rates			
Maximum volumetric PO_4 uptake rate	$r_{PO4\_PP,Ox}$	P-mmol L^{-1} h^{-1}	3.12
Maximum specific PO_4 uptake rate	$q_{PO4\_PP,Ox}$	P-mol C-mol^{-1} h^{-1}	0.038
Volumetric aerobic NH_4 consumption rate[c]	$r_{NH4\_Bio,Ox}$	N-mol L^{-1} h^{-1}	0.15
Maximum specific biomass growth rate[d]	$q_{PAO,Ox}$	C-mol C-mol^{-1} h^{-1}	0.009

[a] Excluding the secondary P release by multiplying $r_{PP\_PO4,Sec,An}$ by the duration of the anaerobic phase (0.063 P-mmol L^{-1} h^{-1} · 2.25 h = 0.142 P-mmol).
[b] Estimated by dividing the maximum volumetric conversion rates by the active biomass concentration at the beginning of the test of 81.4 C-mmol.
[c] Estimated: the aerobic consumption of NH_4 divided by the duration of the aerobic phase.
[d] Estimated: the volumetric aerobic NH_4 consumption rate divided by the N-content of the biomass (0.20 N-mol) and the initial active biomass concentration (81.4 C-mmol L^{-1}).

As observed in Table 2.2.12, the anaerobic stoichiometry, in particular the net P-released/Ac uptake ($Y_{Ac\_PO4,An,}$ PO_4/Ac or P/C) ratio of 0.57 P-mol C-mol^{-1} in combination with the glycogen consumption to Ac uptake ratio of 0.57 C-mol C-mol^{-1} indicates that the observed biomass activity (physiology) corresponds to that dominated by PAO metabolism (Table 2.2.3). This can be confirmed by the relatively low PHV production/PHB production ($Y_{PHV/PHB,An}$) ratio of 0.12 since PHV/PHB ratios close to or lower than 0.10 are commonly observed in PAO-enriched systems (Smolders *et al.,* 1994a) because of the lower glycogen consumption for Ac uptake when compared to GAO-dominated systems (Zeng *et al.,* 2003a). Furthermore, when comparing the observed PO_4/Ac ratio with the values reported in Table 2.2.3 (for enriched EBPR cultures) and

Figure 2.2.5, it appears that PAOs (or their activity) are dominant in the sludge. However, this is a mere rough estimation and should be cross-checked with the use of molecular techniques (chapters 7 and 8). The P/TSS ratio of the sludge can be also used to assess the expected PO_4/Ac ratio (Figure 2.2.7). Nevertheless, based on the previous data, the sludge sample shows a satisfactory EBPR activity.

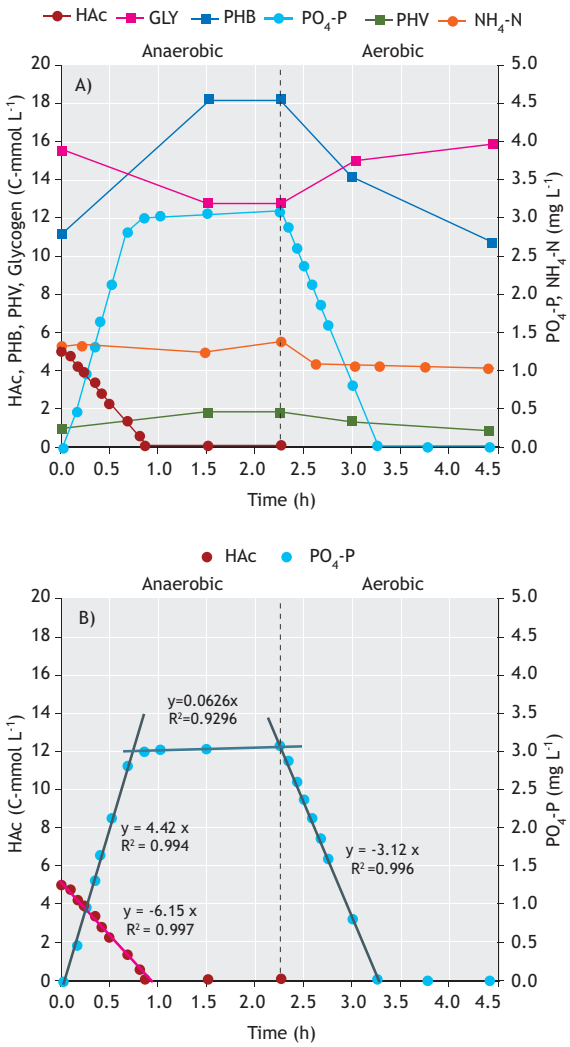

Figure 2.2.8 Graphic representation of the data obtained in the example of the experimental implementation plan for the execution of a batch activity test (Test EBPR.AER.2, Table 2.2.10) performed with a lab-enriched EBPR sludge at 10 °C using synthetic influent at pH 7.0: A) profiles of the experimental data of interest; and, B) experimental data of Ac and PO_4 showing the main trend lines of the conversion rates for the further estimation of the maximum kinetic rates.

An important tool to assess the data consistency and quality is the COD balance. In this example, a COD balance performed to assess the anaerobic EBPR conversions shows that about 5.83 C-mmol Ac and 2.98 C-mmol of glycogen are consumed, while 7.80 C-mmol PHAs are produced. Using the COD conversion factor of 32 mg COD C-mmol^{-1} for both Ac and glycogen and that of 36 mg COD mg C-mmol^{-1} for PHAs, a COD balance error (ΔCOD(%)) of about 0.2 % is estimated using Eq. 2.2.2. A similar COD balance can be performed with the aerobic COD conversions if a respirometry test is performed as indicated in Chapter 3.

Regarding the anaerobic kinetic rates, the $q_{Ac,An}$ and $m_{PP\_PO4,An}$ values of 0.075 C-mol C-mol^{-1} h^{-1} and 7.69 × 10^{-4} P-mol C-mol^{-1} h^{-1}, respectively, appear to be lower than the typical values reported for PAO-enriched systems of around 0.20 C-mol C-mol^{-1} h^{-1} and 2.1 × 10^{-3} P-mol C-mol^{-1} h^{-1}, correspondingly (Table 2.2.12). However, one should realize that the batch activity test was conducted at a temperature of 10 °C, whereas previous values reported in literature have mostly been obtained from tests performed at 20 °C. Thus, for comparison purposes with tests executed at 20 °C (and also at other temperatures), the temperature Arrhenius coefficients (θ) proposed by Meijer (2004) can be applied (of 1.094 for carbon uptake and 1.071 for anaerobic maintenance) to estimate the equivalent kinetic rates at 20 °C based on the tests performed at 10 °C. Therefore, the equivalent kinetic rates at 20 °C are: 0.18 C-mol C-mol^{-1} h^{-1} for the maximum acetate uptake rate (estimated as $q_{Ac,20,An} = q_{Ac,10,An} / \theta^{(10-20)}$) and 1.53 × 10^{-3} P-mol C-mol^{-1} h^{-1} ($m_{PP\_PO4,20,An} = m_{PP\_PO4,10,An} / \theta^{(10-20)}$).

Similarly, for the maximum aerobic specific uptake rate ($q_{PO4\_PP,Ox}$) of 0.038 P-mol C-mol^{-1} h^{-1} observed at 10 °C, an equivalent maximum specific rate of 0.050 P-mol C-mol^{-1} h^{-1} is estimated at 20 °C which is within previous reported values in literature (Table 2.2.6). As previously mentioned, biomass growth cannot be directly determined from the increase in MLVSS since the potentially low biomass increase will probably fall into the standard error of the MLVSS analytical determination technique. Instead, it is estimated based on the NH_4 consumption observed in the test (ensuring that NH_4 is not removed by any other biological or chemical process). Thus, a maximum specific biomass growth rate ($q_{Bio,Ox}$) of 0.009 C-mol C-mol^{-1} h^{-1} (C-mol new biomass produced per C-mol of existing biomass) is determined. After re-calculating the approximate biomass growth rate from 10 °C at 20 °C (using the Arrhenius temperature coefficients for biomass growth of 1.081), the estimated biomass growth rate is 0.020 C-mol C-mol^{-1} h^{-1}, which is

in the range of previously reported values for EBPR sludge (0.016 C-mol C-mol^{-1} h^{-1}) (Smolders, 1995). Overall, the stoichiometric and kinetic parameters obtained in the test are comparable to similar values previously reported in literature for EBPR cultures (Table 2.2.3), strongly indicating that the EBPR activity observed in the test was typical of an enriched EBPR culture.

2.2.8 Additional considerations

2.2.8.1 GAO occurrence in EBPR systems

Due to the harmful effects that they can have on EBPR performance, the occurrence of GAOs has been the subject of extensive research in past years. So far, (*i*) the availability of a sole COD source in the influent (either acetate or propionate), (*ii*) a temperature higher than 20 °C, and (*iii*) pH values lower than 7.0 have been suggested as key factors favouring the presence of GAOs in (lab-scale) EBPR systems (Filipe *et al.,* 2001b; Oehmen *et al.,* 2004; Lopez-Vazquez *et al.,* 2009b). The most characteristic aspects that suggest the presence of GAOs are anaerobic P-released/C-uptake ratios much lower than 0.50 P-mol C-mol^{-1} and incomplete aerobic PO_4 uptake. Despite the apparently frequent occurrence of GAOs in lab-scale EBPR systems (possibly as a consequence of operating the lab-scale systems at the boundaries of the aforementioned parameters: with a single carbon source, usually acetate, at pH 7.0 and 20 °C), abundant GAO populations have rarely been found in full-scale municipal EBPR systems (Thomas *et al.,* 2003; Saunders *et al.,* 2003; Lopez-Vazquez *et al.,* 2008a; Lopez-Vazquez, 2009; Kong *et al.,* 2006), unless certain particular conditions (such as the discharge of industrial effluents) take place (Burow *et al.,* 2007). Although a low anaerobic P-released/C-uptake ratio could suggest a higher activity or involvement of GAOs, as described in the text below, recent observations have provided evidence that PAOs, under limiting intracellular poly-P conditions, may be able to perform a similar metabolism like GAOs under anaerobic conditions but still achieve aerobic full P-removal (Schuler and Jenkins, 2003; Zhou *et al.,* 2008; Acevedo *et al.,* 2012; Welles *et al.,* 2015b). Therefore, an anaerobic P-released/C-uptake ratio considerably lower than 0.50 P-mol C-mol^{-1} (e.g. around 0.35 P-mol C-mol^{-1}) observed in EBPR batch activity tests does not strictly imply that GAOs are more abundant than PAOs. While the actual mechanisms influencing the use of a GAO-type metabolism by PAOs will probably continue to be a matter of future research efforts, the use of microscopic and molecular techniques (chapters 7 and 8) is strongly recommended to identify the dominant EBPR microbial population.

2.2.8.2 The effect of carbon source

It is well known that RBCOD fed to the anaerobic stage (containing mainly volatile fatty acids such as acetate and propionate) enhances the growth of EBPR biomass (Comeau *et al.,* 1986; Mino *et al.,* 1998; Oehmen *et al.,* 2004). Other RBCOD sources, such as glucose, are not suitable since they appear to enhance the growth of GAOs or G-bacteria (Cech and Hartman, 1993). Also, it is assumed that more complex substrates need to be hydrolysed and fermented to VFA to become available to EBPR biomass (Wentzel *et al.,* 2008). In this regard, it is likely that more complex COD sources will not be fully consumed in the anaerobic stage and will 'leak' into the anoxic or aerobic stages, influencing the EBPR anoxic and aerobic results (for instance, when executing combined anaerobic-anoxic-aerobic tests, such as Tests EBPR.ANOX.2 or EBPR.AER.2). Ideally, for a satisfactory interpretation of the experimental data obtained, no COD source may be present in the anoxic or aerobic stage of an EBPR batch activity test (unless it is also a subject of interest). In addition, recent developments hypothesize that, besides the apparently known PAOs (*Candidatus Accumulibacter phosphatis*), other organisms, such as *Actinobacteria* or S-PAOs (Kong *et al.,* 2005; Wu *et al.,* 2014), are able to perform an excessive P-uptake like *Accumulibacter* using alternative electron donors (e.g. aminoacids or H_2S). For day-to-day or regular tests on well-known EBPR systems, the use of synthetic media containing VFA can be good enough to provide a satisfactory assessment of EBPR activity, as presented in this chapter. However, the use and application of more complex COD sources, which may be undoubtedly present in raw or settled municipal wastewater, can lead to either sub-optimal EBPR activity or (yet to be known) different EBPR metabolisms. The latter has been and will continue to be a matter of extensive research. Nevertheless, the reader should be aware that such conditions can lead to results that may differ from those presented in this chapter.

2.2.8.3 The effect of temperature

While extensive research has been advocated to assess the temperature dependencies of EBPR cultures, suggesting that temperatures lower than 20 °C enhance the growth of PAOs whereas higher temperatures favour the development of GAOs (Brdjanovic *et al.,* 1997, 1998b; Lopez-Vazquez *et al.,* 2009a), certain

observations have indicated that stable EBPR systems can operate at temperatures higher than 25 °C (Cao *et al.,* 2009). Though case-specific combinations between wastewater composition, operating and environmental conditions will play a major role, the long-term operation and acclimatization of EBPR cultures to those particular conditions can also lead to the development and enrichment of PAO cultures (or similar organisms sharing the PAOs' phenotype) capable of performing stable EBPR at a higher temperature. In this regard, the identification of these organisms is of major importance where the methods and techniques presented in Chapter 7 and particularly in Chapter 8 will be needed and applicable to elucidate the identity of these organisms.

2.2.8.4 The effect of pH

As discussed, pH has a direct influence on EBPR cultures (Smolders *et al.,* 1994a, Filipe *et al.,* 2001a). During EBPR batch activity tests, it should be carefully monitored and well-controlled to obtain reliable data (avoiding pH fluctuations higher than ± 0.1-0.2). However, higher pH levels (particularly above pH 8.0) combined with the presence of RBCOD can lead to (expectedly) higher anaerobic P release and favour chemically-induced calcium phosphate precipitation or even struvite formation depending on the wastewater composition (NH_4MgPO_4 or $KMgPO_4$) (Lin *et al.,* 2012; Mañas *et al.,* 2011). Also, it cannot be disregarded that the presence of aluminum salts in the wastewater produced by the discharge of drinking water sludge can play a role and lead to the precipitation of phosphorus, particularly if there is enough retention time in the sewer network. These processes will reduce the biological availability of phosphorus for PAOs, resulting in the potential deterioration of the EBPR process as PAOs will not be able to replenish their intracellular poly-P pools. Such conditions will lead to different values to those presented in this chapter. On the other hand, although the previous process may not be desirable and, thus, should be avoided in continuous conventional alternating anaerobic-anoxic-aerobic EBPR systems, it can offer interesting alternative options for P recovery that may be worth exploring in view of the essential role of phosphorus in the food chain and the potential depletion of the conventional world's phosphorus sources.

2.2.8.5 Denitrification by EBPR cultures

Denitrifying EBPR sludge has been the subject of extensive debate since the 1990s. While satisfactory simultaneous NO_3 removal and PO_4 uptake has been observed in the anoxic stage of several EBPR systems (Vlekke *et al*., 1988; Kuba *et al.,* 1993, 1996, 1997a, 1997b; Wachtmeister *et al.,* 1997; Brdjanovic *et al.,* 2000; Zeng *et al.,* 2003b), limited or inconsistent simultaneous denitrification and PO_4 uptake has been observed in other lab- and full-scale studies (Kerrn-Jespersen and Henze, 1993; Hu *et al.,* 2003; Carvalho *et al.,* 2007; Lopez-Vazquez *et al.,* 2008a). Actually, the discovery of the existence of two different *Accumulibacter* clades (known PAOs) seemed to have been encouraged by assessing the denitrifying capabilities of EBPR biomass (Flowers *et al.,* 2008). Flowers *et al.* (2008) proposed that the so-called *Accumulibacter* clade Type I has a full-denitrifying capability (able to denitrify from nitrate to di-nitrogen gas), whereas *Accumulibacter* clade Type II appears to be only able to denitrify from nitrite onwards (in line with the first observations drawn by Kerrn-Jespersen and Henze, 1993). As suggested by Kuba *et al.* (1996) and Lopez-Vazquez *et al.* (2008a), the simultaneous NO_3 removal and anoxic PO_4 uptake capability of an EBPR sludge may be a reflection of both the induction of the required denitrifying enzymes (nitrate and nitrite reductase) and the development of EBPR and side populations able to denitrify. Therefore, when executing anoxic EBPR activity tests, the relative anoxic EBPR activities observed can vary widely from practically zero to considerably high anoxic activities (Table 2.2.8, Table 2.2.9) as a function of the previously discussed exposure to anoxic conditions and the development of the denitrifying populations in the lab- or full-scale systems.

2.2.8.6 Excess/shortage of intracellular compounds

Although the depletion of the intracellular poly-P pools was initially assumed to be a limiting factor for anaerobic VFA uptake by PAOs (Brdjanovic *et al.,* 1997), later developments showed that PAOs can utilize higher amounts of intracellularly stored glycogen as an energy source for anaerobic VFA uptake to compensate for the limiting poly-P availability, and still perform satisfactory fully aerobic P-uptake (Schuler and Jenkins, 2003; Zhou *et al.,* 2008, Acevedo *et al.,* 2012; Welles *et al.,* 2014, 2015b, 2016, submitted). This is reflected in anaerobic P-released/C-uptake ratios much lower than 0.50 P-mol C-mol^{-1} (Figure 2.2.7) and higher anaerobic glycogen/C ratios (Table 2.2.3). Based on observations from Schuler and Jenkins (2003) and Welles *et al.* (2015b), the shift from the metabolic utilization of poly-P to glycogen appears to take place as soon as the biomass total P/TSS ratio drops below 0.08 mg P g TSS^{-1} (Acevedo *et al.,* 2014). Furthermore, Welles *et al.* (2015b) have observed

that the maximum kinetic rates of the two known *Accumulibacter* clades (Flowers *et al.,* 2008) appear to be affected in a different manner depending on the intracellular poly-P availability, with *Accumulibacter* Type I more affected than *Accumulibacter* Clade Type II. This can explain potential deviations from those presented in this chapter, which can be supported by the determination of the intracellular poly-P and glycogen contents or at least by the estimation of the biomass P/TSS ratio.

2.2.8.7 Excessive aeration

The exposure of EBPR biomass to extended aeration periods (e.g. longer than 12-24 h) can lead to the sequential utilization of PHAs, glycogen and intracellular poly-P under aerobic conditions (Brdjanovic *et al.,* 1998c; Lopez *et al.,* 2006), as a consequence of the biomass needs to cover their aerobic maintenance requirements. Consequently, it will lead to an eventual aerobic P release as soon as the intracellularly stored poly-P pools start to be hydrolysed. Thus, if EBPR sludge samples are aerated for extensive periods of time prior to the execution of the batch activity tests (e.g. overnight or during transportation), it is likely that a lower EBPR activity will be observed due to the potentially low(er) poly-P and glycogen contents of the sludge. Therefore, over-aeration conditions should be avoided. Furthermore, it deserves particular attention for the satisfactory operation of full-scale activated sludge EBPR systems because excessive aeration periods during low loading conditions (e.g. weekends or holidays periods) can result in undesired aerobic P release, affecting the treated effluent quality.

2.2.8.8 Shortage of essential ions

Though it may be trivial, the presence of macro- and micro-nutrients in the right concentration and (bio-) availability is essential for EBPR activated sludge systems. For instance, potassium, magnesium, iron, and calcium, among others, are rather important to regulate the microbial EBPR metabolism and support the storage of intracellular compounds (Brdjanovic *et al.,* 1997; Burow *et al.,* 2007; Barat *et al.,* 2008). Their absence, for instance of potassium, can lead to the deterioration of the EBPR process (Brdjanovic *et al.,* 1997), but their excess can influence the metabolism of PAOs inducing a GAO metabolism (Jobaggy *et al.,* 2006; Barat *et al.,* 2008), possibly due to the chemical precipitation of phosphorus with the aforementioned elements. This will reduce their bio-availability and therefore the aerobic replenishment of poly-P pools. The absence or presence in excessive concentrations of the aforementioned elements should be checked if one suspects that their concentrations differ from those regularly observed in municipal wastewater treatment systems.

2.2.8.9 Toxicity/inhibition

A limited number of compounds have been identified to be toxic or inhibiting to the EBPR process. The presence of nitrate or nitrite in the anaerobic zone is considered harmful for the EBPR process since they enhance the activity of ordinary denitrifying organisms that can consume the available RBCOD to the detriment of PAOs (Wentzel *et al.,* 2008). Also, the presence of nitrite in the aerobic zone in concentrations as low as 6-8 mg L^{-1} has been proven to inhibit PAOs (Saito *et al.,* 2004), which can worsen the negative effects at lower pH due to the increase in free nitrous acid (FNA) (Zhou *et al.,* 2008; Pijuan *et al.,* 2010). The latter favours the occurrence of GAOs over PAOs since GAOs appear to be more tolerant to the presence of FNA (Pijuan *et al.,* 2011), resulting in the deterioration of the EBPR process. Salinity is another factor that may inhibit the activity of PAOs in the short-term (hours) (Welles *et al.,* 2014, 2015a). Welles *et al.* (2014) observed that, after a short-term exposure (hours), chloride concentrations as low as 10,000 mg NaCl L^{-1} (1 % salinity) can lead to more than 50 % inhibition of the anaerobic metabolism of PAOs and practically fully inhibit the aerobic metabolism. Similar circumstances can take place due to a sudden saline intrusion into the sewage or saline intrusion to the plant (particularly in coastal regions), or caused by industrial effluent discharges. Nevertheless, the long-term exposure of EBPR sludge to high salinity concentrations (> 35,000 mg NaCl L^{-1}, 3.5 % salinity) can enhance the acclimatization of EBPR biomass which can become salt-tolerant and be able to perform satisfactory EBPR at salinity concentrations equivalent to those observed in seawater. Another compound that is potentially inhibitory or toxic to EBPR sludge is H_2S, which may be formed in the sewage (due to saline intrusion or industrial discharges) or in the anaerobic stage of the EBPR activated sludge system. Its presence can be rather inhibitory to the anaerobic metabolism of PAOs at concentrations as low as 20-25 mg H_2S L^{-1} leading to 50 % inhibition (Saad *et al.,* 2013; Rubio-Rincon *et al.,* 2016, submitted). Overall, inhibiting effects will be reflected in limiting or sub-optimal EBPR activity. If feasible, the sludge can be washed in a mineral solution (as explained in Section 2.2.3.5) to avoid the potentially inhibiting or toxic compounds.

2.3 BIOLOGICAL SULPHATE-REDUCTION

2.3.1 Process description

Sulphate (SO_4^{2-}) is naturally present in surface water and ground water and depending on the geographical location, its concentrations in drinking water supply networks can vary. Consequently, the presence of other sulphur compounds namely organic sulphides, including mercaptans, dimethyl sulphides and dimethyl disulphides is also very common in domestic wastewater. The concentration of sulphate in domestic sewage usually ranges from 20 to 60 mg L^{-1} (Moussa *et al*., 2006). However, the sulphate concentration in sewage can reach as high as 500 mg L^{-1}, due to the discharge of sulphate-rich industrial effluents, seawater-based toilet flushing or intrusion of saline water into the sewer (Lens *et al*., 1998; Chen *et al*., 2010; Ekama *et al*., 2010).

The metabolism of sulphate-reducing bacteria (SRB), beside its applications in domestic sewage treatment, can be exploited beneficially in specific industrial effluent treatment processes that generate sulphate-rich wastewater (Lens *et al*., 1998; Muyzer and Stams, 2008). Such industries are, among others, potato starch production, pulp and paper mills, food and fermentation industries and seafood processing facilities. In wastewater treatment applications, sulphate is usually completely reduced to sulphide, as this conversion yields the highest free Gibb's energy ($\Delta G^{\circ'}$, Table 2.3.1).

Table 2.3.1 Sulphate transformation reactions performed by SRB (Jørgensen, 2006; Liamleam and Annachhatre, 2007).

Reactions	$\Delta G^{\circ'}$ (KJ mol^{-1})
$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O$	-152.2
$SO_4^{2-} + H_2 + 2H^+ \rightarrow HSO_3^- + H_2O$	+19.7
$HSO_3^- + 3H_2 \rightarrow HS^- + 3H_2O$	-171.7
$3HSO_3^- + H_2 + H^+ \rightarrow S_3O_6^{2-} + 3H_2O$	-46.3
$S_3O_6^{2-} + H_2 \rightarrow S_2O_3^{2-} + HSO_3^- + H^+$	-123.0
$S_2O_3^{2-} + H_2 \rightarrow HS^- + HSO_3^-$	-2.1
$SO_4^{2-} + 2H^+ + ATP \rightarrow APS + PP_i$	+46.0
$PP_i + H_2O \rightarrow 2P_i$	-21.9
$APS + H_2 \rightarrow HSO_3^- + AMP + H^+$	-68.0

Dissimilatory sulphate-reduction is the most important anaerobic process in many different environments (Balk *et al*., 2008). The initial step of biological sulphate-reduction involves the transfer of exogenous sulphate through the bacterial cell membrane into the cell. The sulphate dissimilation process proceeds via the action of adenosine triphosphate (ATP) sulphurylase (Figure 2.3.1). ATP produces the highly activated molecule adenosine phosphosulphate (APS), and pyrophosphate (PPi) in the presence of sulphate, which yields inorganic phosphate. Further, APS is rapidly converted to bisulphite (HSO_3^-) by the cytoplasmic enzyme APS reductase. Pyrophosphate is hydrolyzed and the sulphate moiety of APS is reduced to bisulphite, together with adenosine monophosphate (AMP). Bisulphite in turn may be reduced via a number of intermediates to form the sulphide ion. Bisulphite is reduced to bisulfide (HS^-) via bisulphite reductase. By another mechanism, bisulphite reduction via the enzymes bisulphite reductase, trithionate reductase and thiosulfate reductase yields trithionate ($S_3O_6^{2-}$) and thiosulfate ($S_2O_3^{2-}$) as free intermediates. The physiology and growth of these bacteria has been studied in depth and is well documented (Cypionka, 1987; Gibson, 1990; Hansen, 1994; Rabus *et al*., 2006).

The organisms responsible for the reduction of sulphur compounds belong to both bacteria and prokaryotes (Postgate, 1965; Muyzer and Stams, 2008); nonetheless, in literature, the term SRB is used. In this chapter, the term SRB includes both bacteria and prokaryotes. The bacterial sulphate reducers are categorized into different branches, the *Deltaproteobacteria* with more than 25 genera, the Gram-positive bacteria that include *Desulfotomaculum* and *Thermodesulfobium* and Gram-negative sulphate reducers that include *Thermodesulfobacterium* and *Thermodesulfatator* (Mori *et al*., 2003; Moussard *et al*., 2004; Balk *et al*., 2008). In general, SRB are found present and active in sewerage systems and wastewater treatment plants. Several authors have also reported SRB activities in freshwater, marine, hypersaline and oil/hydrocarbon polluted sites (Cravo-Laureau *et al*., 2004; Almeida *et al*., 2006; Kjeldsen *et al*., 2007).

SRB are facultative anaerobes that live in oxygen-free or depleted environments and utilize sulphate as a terminal electron acceptor to produce hydrogen sulphide (H_2S) as one of its metabolic end products. SRB can survive under extreme environmental and operating conditions over a rather wide range of pH (4.0 to 9.5), temperature (25-75 °C) and pressures of up to 500 atm (Madigan *et al*., 2009; Tang *et al*., 2009). Sulphate is redox sensitive and the production of sulphide is an indicator of SRB activity which depends on several factors including sulphate concentrations, the

concentration of organic matter/nutrients, pH and temperature, among others. In sewerage and municipal wastewater treatment plants, the presence of SRB is considered to be undesirable, due to corrosion and instability in methanogenic activity, causing an insufficient digestion process (Oude Elfrenink *et al*., 1994). SRB have been recognized as the major microbiologically-influenced corrosion-causing bacteria in oil/gas pipelines and sewer systems (Al Abbas *et al*., 2013). Microbiologically-influenced corrosion is aggravated due to the synergistic interaction of different microbes such as iron and manganese-reducing bacteria, carbon dioxide-reducing bacteria that co-exist and through cooperative metabolism with SRB (Little and Lee, 2007). According to Kjeldsen *et al*. (2004), the presence of SRB in activated sludge is of interest because sulphate-reduction can have negative effects on the wastewater treatment plant operation. Other negative side-effects of the activity of SRB is that the produced sulphide will inhibit other main microorganisms involved in the treatment process, such as methanogenic bacteria (MET), phosphorus-accumulating organisms (PAO), nitrifiers and others. Exceedingly high sulphide levels can be toxic for microorganisms performing methanogenesis and sulphate-reduction. High sulphide concentration can also have a deteriorating effect on the activated sludge floc structure, for instance, deflocculation, by reducing Fe(III) to Fe(II) as FeS (Caccavo *et al*., 1996; Nielsen and Keiding, 1998). The authors attributed this phenomenon to the better flocculating properties of Fe(III) rather than Fe(II), mainly due to its valance and lower solubility.

On the other hand, sulphide production can lead to the growth of filamentous bacteria and result in concomitant sludge bulking (Yamamoto *et al*., 1991; Zeitz *et al*., 1995; Kjeldsen *et al*., 2004).

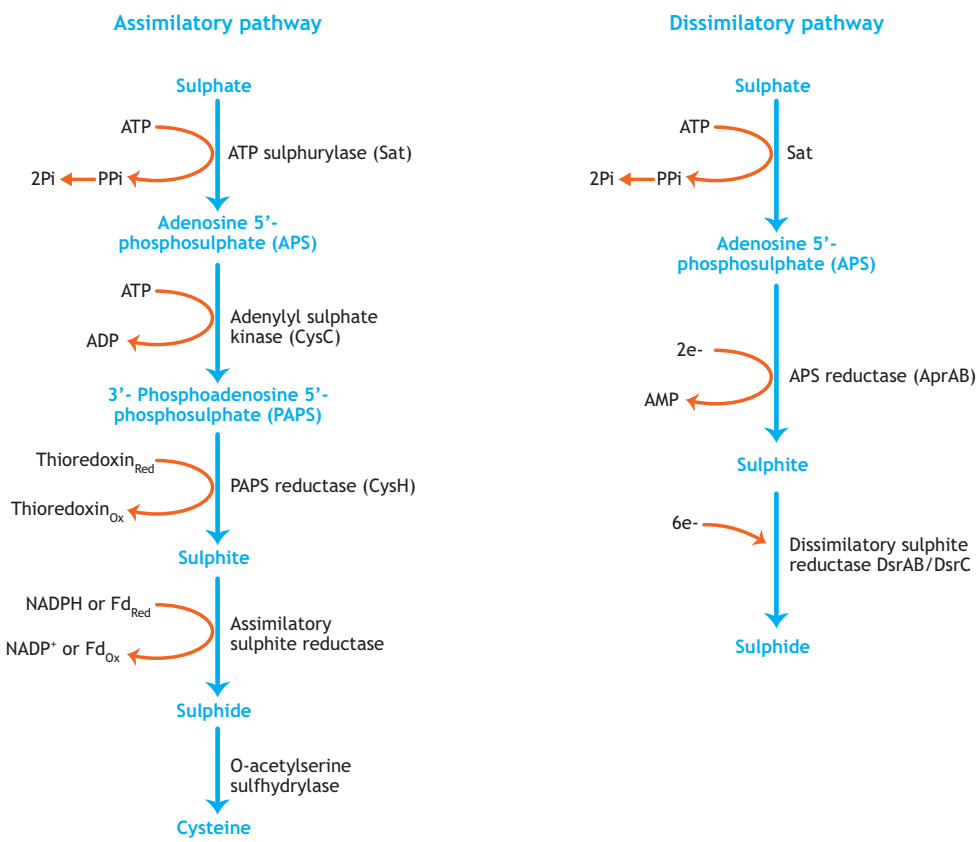

Figure 2.3.1 Prokaryotic assimilatory and dissimilatory pathways for sulphate-reduction (Grein *et al*., 2013).

Driven by the potable water supply stress mitigated by using seawater for toilet flushing, the research group of Hong Kong University of Science and Technology (HKUST) recognized the benefits of sulphate present in saline sewage and successfully developed a process that takes a big advantage of sulphate and SRB: the SANI® process (Sulphate-reduction, Autotrophic denitrification and Nitrification Integrated process); the only technology that applies SRB for municipal wastewater treatment purposes. Its application has led to lower sludge production, higher coliform (pathogen) and heavy metal removal while using less space and energy (Wang *et al.*, 2009; Abdeen *et al.*, 2010). In addition, SRB-based processes have been used as a pre-treatment step to enhance the digestion process. Recently, Daigger *et al.* (2015) tested a pilot-scale membrane bioreactor (MBR) to remove elemental sulphur from an anaerobically pre-treated pulp and paper effluent containing high concentrations of dissolved sulphide. Although SRB play a major role in treatment plants, they are not frequently studied in domestic wastewater treatment. To understand the positive and negative effects of SRB activity in a domestic wastewater treatment plant, activity tests should be conducted. Batch tests can be useful to estimate to what extent SRB are present and active in sewage and treatment plants and to provide an insight to develop measures that can stimulate or suppress SRB activity, depending on the chosen process design and operational conditions. Therefore, from a process performance perspective, the stability and continuation of the treatment process is highly dependent on (among others) the concentration of the sulphide (and pH) present in the liquid phase.

2.3.2 Sulphide speciation

Sulphide can be present in wastewater in various states (H_2S, HS^- and S^{2-}). The unionized H_2S is known to have the strongest inhibitory effect, due to its ability to permeate the cell membrane. pH is the main factor that determines the proportion of S^{2-} present in wastewater. Figure 2.3.2A indicates the relation between pH and sulphide speciation. As the pH of wastewater is usually around 7.6, sulphide will mostly be present as HS^-. The Eh-pH diagram depicted in Figure 2.3.2B shows the dominant aqueous species and stable solid phases on a plot defined by the Eh and pH axes. It is noteworthy that, under anaerobic conditions, the zero valent dissolved and suspended sulphur may exist in aqueous solutions as colloidal sulphur or as metal-ligated or free polysulphides and hydropolysulphides (Kamyshny *et al.*, 2008).

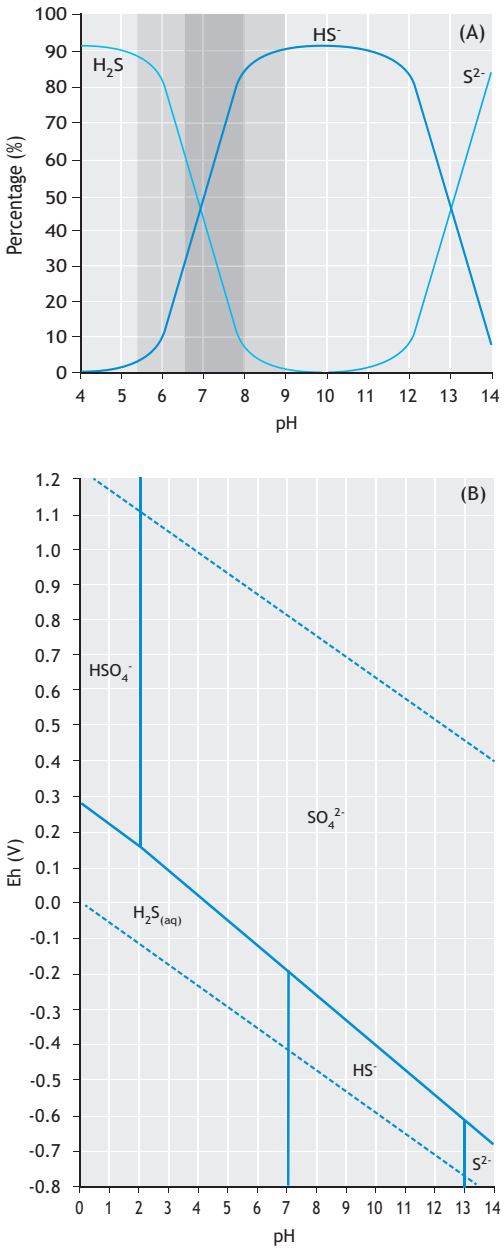

Figure 2.3.2 Sulphide speciation in wastewater: (A) pH influence at 25 °C, as adapted from Rintala and Puhakka (1994); and (B) Eh-pH diagram of various S species in aqueous solution (FACT database, adapted from Bale *et al.*, 2002).

Sulphide speciation can also be influenced by the partition coefficient value, i.e. the ratio of concentration between the gas phase and the liquid phase, which is also heavily pH and temperature-dependent. Other parameters which might influence sulphide speciation are temperature and salt concentration.

The distribution of sulphide in the gas phase (g) and liquid phase (l) can be represented by the following equation (Hulshoff Pol *et al*., 1998):

$$S_{H2S} = \alpha \cdot C_{H2S} \qquad \text{Eq. 2.3.1}$$

Where, α (alpha) is the dimensionless distribution coefficient for H_2S liquid-gas phase equilibrium. In the liquid phase, S_{H2S}, H_2S exists as uninonized H_2S or in its ionized forms (as bisulfide, HS^- or sulphide, S^{2-}). Even small variations in the pH value will significantly affect the free H_2S concentrations. Again, the Henry's law constant for H_2S at 25 °C is ~3.4 mg L^{-1} atm^{-1}, which indicates the large volatility of this species. The dependence of α on the temperature and pKa values (pKa = - log Ka) is shown in Figure 2.3.3.

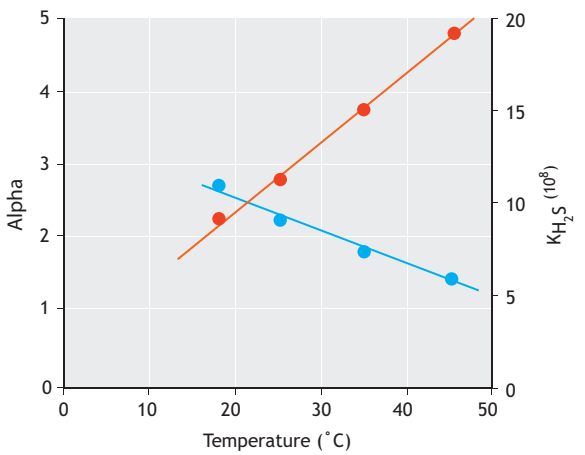

Figure 2.3.3 Temperature dependency on the distribution coefficient alpha (●) and dissociation constant pKa (●) of H_2S (adapted from Hulshoff Pol *et al*., 1998).

2.3.3 Effects of environmental and operating conditions on SRB

2.3.3.1 Carbon source

To obtain energy for growth and maintenance, SRB can oxidize a wide range of substrates acting as the electron donor that include, among others, hydrogen, alcohols, fatty acids, aromatic and aliphatic compounds (Liamleam and Annachhatre, 2007), while sulphate is used as an external electron acceptor. In certain (industrial) plants, when the wastewater contains an insufficient electron donor/carbon source and there is interest in promoting the removal of organics by SRB, an external carbon source is usually added. Liu and Peck (1981) determined appropriate electron donors for SRB when grown as a pure culture. Several electron donors have been studied as energy and carbon sources for SRB (Table 2.3.2).

Table 2.3.2 Compounds used as energy substrates by SRB (adapted from Hansen, 1993).

Compound	Substrate
Inorganic	Hydrogen, carbon monoxide, etc.
Monocarboxylic acids	Lactate, acetate, butyrate, formate, propionate, isobutyrate, 2- and 3- methylbutyrate, higher fatty acids up to C18, pyruvate.
Dicarboxylic acids	Succinate, fumarate, malate, oxalate, maleinate, glutarate, pimelate.
Alcohols	Methanol, ethanol, propanol, butanol, ethylene glycol, 1,2- and 1,3-propanediol, glycerol.
Amino acids	Glycine, serine, cysteine, threonine, valine, leucine, isoleucine, aspartate, glutamate, phenylalanine.
Miscellaneous	Choline, furfural, oxamate, fructose, benzoate, 2-, 3- and 4-OH-benzoate, cyclohexanecarboxylate, hippurate, nicotinic acid, indole, anthranilate, quinoline, phenol, p-cresol, catechol, resorcinol, hydroquinone, protocatechuate, phloroglucinol, pyrogallol, 4-OH-phenyl-acetate, 3-phenylpropionate, 2-aminobenzoate, dihydroxyacetone

Most electron donors are products of fermentation, monomers or cell components from other sources. The three major criteria for selecting a suitable electron donor for sulphate-reduction process are: (*i*) efficiency of sulphate removal in the effluent complemented by a low COD, (*ii*) the electron donor availability, and (*iii*) cost of unit sulphate converted to sulphide (van Houten *et al*., 1996). With greater interest in the development of sustainable SRB technologies, hydrogen has been identified as one of the most important substrates for SRB. *Desulfovibrio* species have a high affinity for hydrogen, and this is considered to be the reason why they are able to out-compete hydrogenotrophic methanogens in sulphate-rich environments (Widdel, 2006).

Recently used energy sources for biological sulphate-reduction for industrial applications include complex organic carbon sources. Van Houten *et al*. (1996) used synthetic gas (mixtures of H_2, CO, and CO_2) as the energy source in laboratory scale gas-lift reactors and showed

biological sulphate-reduction by SRB. Numerous organic waste matrices (sources) have also been used as carbon sources and electron donors. These include leaf mulch, wood chips, sewage sludge, sawdust, compost, animal manure, whey, vegetable compost, and other agricultural wastes (Liamleam and Annachhatre, 2007). Lactate and molasses, though cost-effective, are not completely oxidized by SRB, generating high COD in the effluent. Hydrogen and ethanol, despite being more expensive, are still being used for sulphate loads greater than 200 kg SO_4^{2-} h^{-1}. However, due to safety reasons, ethanol is preferred to hydrogen. Interestingly, one of the first carbon sources to be considered was waste sewage sludge (Butlin *et al*., 1956) and COD is usually present in relatively higher concentrations in municipal wastewater, suggesting that for municipal wastewater treatment applications no external electron donors may be needed. During the fermentation of COD in the sewerage system or in the anaerobic zones of wastewater treatment plants, volatile fatty acids (VFA) such as acetate and propionate may contribute to most of the readily biodegradable COD (RBCOD). Other microorganisms like methanogens or acetogens can also utilize VFA as a carbon source under anaerobic conditions, competing against SRB for VFA. The interactions between SRB, methanogens and acetogens depend on the type of VFA and other substrates present in the wastewater (Figure 2.3.4).

From the equations and Gibb's free energy values (ΔG°′) shown in Table 2.3.3, it can be observed that SRB can utilize a broad range of carbon sources (lactate, hydrogen, acetate, propionate and butyrate), whereas methanogens rely mostly on hydrogen and acetate, while acetogens can utilize lactate, propionate and butyrate. Thus, SRB will compete against acetogens for lactate, propionate and butyrate, and against methanogens for hydrogen and acetate. Overall, SRB can have bigger advantages over other anaerobic organisms, as long as there is sufficient sulphate available.

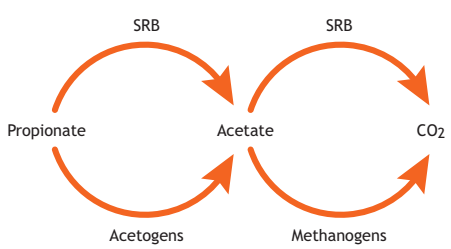

Figure 2.3.4 The competition between sulphate-reducing bacteria (SRB), methanogens and acetogens for VFA in municipal wastewater.

Table 2.3.3 Typical reactions for sulphur-reducing bacteria, methanogens and acetogens: ΔG°′ (kJ mol^{-1}) values are adapted from Thauer *et al.* (2007).

Reaction	ΔG°′ (kJ mol^{-1})
Sulphate-reducing bacteria	
$Lactate^- + 0.5SO_4^{2-} \rightarrow Acetate^- + HCO_3^- + 0.5HS^-$	-80.2
$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$	-36.4
$Acetate^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6
$1.33Propionate^- + SO_4^{2-} \rightarrow 1.33Acetate^- + 1.33HCO_3^- + 0.75HS^- + 1.33H^+$	-50.3
$2Butyrate^- + SO_4^{2-} \rightarrow 4Acetate^- + HS^- + H^+$	-55.6
Methanogens	
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-33.9
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Acetogens	
$Lactate^- + 2H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	-4.2
$Propionate^- + 3H_2O \rightarrow Acetate^- + HCO_3^- + H^+ + 3H_2$	+71.6
$Butyrate^- + 3H_2O \rightarrow 2Acetate^- + H^+ + 2H_2$	+9.6

2.3.3.2 COD to SO_4^{2-} ratio

As shown in Table 2.3.3, the oxidation of COD is coupled to the reduction of sulphate, in a COD/SO_4^{2-} ratio of 0.67 g COD g SO_4^{-1} (Khanal, 2008). Thus, a complete COD oxidation by SRB can only be achieved if the conditions are not sulphate-limiting (i.e. the COD/SO_4^{2-} ratio is 0.67 or lower). For medium-strength domestic wastewaters (for e.g. soluble COD concentrations of approximately 300 mg COD L^{-1}), the minimal amount of sulphate required in the influent can easily be achieved due to the intrusion of brackish water into the sewer, seawater-based toilet flushing and/or discharge of sulphate-rich (industrial) wastewaters. On the other hand, a higher COD/SO_4^{2-} ratio will favour the growth of methanogenic bacteria that will consume part of the available RBCOD.

2.3.3.3 Temperature

The sulphate-reduction rate of SRB and their doubling time are highly dependent on temperature (Hulshoff Pol *et al*., 1998; Pikuta *et al*., 2000). The doubling time of microorganisms capable of performing intense sulphidogenesis has been observed to drastically vary between 10 and 118 h within a temperature range of 30 to 60 °C (Pikuta *et al*., 2000). Table 2.3.4 shows the optimum temperature for the growth of some SRB.

Table 2.3.4 Temperature range for some SRB (Tang *et al*., 2009).

SRB	Temperature (°C)	
	Range	Optimum
Desulfobacter	28–32	–
Desulfobulbus	28–39	–
Desulfomonas	–	30
Desulfosarcina	33–38	–
Desulfovibrio	25–35	–
Thermodesulforhabdus norvegicus	44–74	60
Desulfotomaculum luciae	50–70	–
Desulfotomaculum solfataricum	48–65	60
Desulfotomaculum thermobenzoicum	45–62	55
Desulfotomaculum thermocisternum	41–75	62
Desulfotomaculum thermosapovorans	35–60	50
Desulfacinum infernum	64	–

The effect of temperature on bacterial sulphate-reduction rates can be evaluated using the Arrhenius model (Isaksen and Jørgensen, 1996). The activation energy for sulphate-reduction can be determined by plotting the logarithm of the sulphate-reduction rate versus the inverse of temperature as follows:

$$\ln(r_{SO4.An}) = \ln(A) + \left(\frac{-E_a}{RT}\right) \qquad \text{Eq. 2.3.2}$$

Where, E_a is the activation energy (J mol^{-1}), $r_{SO4,An}$ is the sulphate-reduction rate (nmol cm^{-3} d^{-1}), Where, E_a is the activation energy (J mol^{-1}), k is the sulphate-reduction rate (nmol cm^{-3} d^{-1}), A is a constant, R is the molecular gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature (K).

2.3.3.4 pH

Another parameter that may influence the competition between SRB and methanogens is pH. pH affects the different metabolic processes of SRB, methanogens and acetogens. Also, pH affects the extent to which VFA and sulphide are inhibitory or toxic to SRB. A slight increase in pH (e.g. from 7.6 to 7.8) helps SRB to become dominant. Both VFA and sulphide tend to be more inhibitory or toxic at low pH values because the protonated species of VFA and total solids (TS) become abundant at these pH and, due to their neutral charge, they can freely diffuse through the membranes of the organisms and affect their intracellular constituents such as enzyme mechanisms. In any case, methanogens are more inhibited by the presence of sulphide than SRB. Therefore, batch activity tests regarding the VFA content, COD/SO_4^{2-} ratio and pH effect on SRB activity are of practical interest. A typical example showing the effect of pH and temperature on the doubling time of SRB is illustrated in Figure 2.3.5.

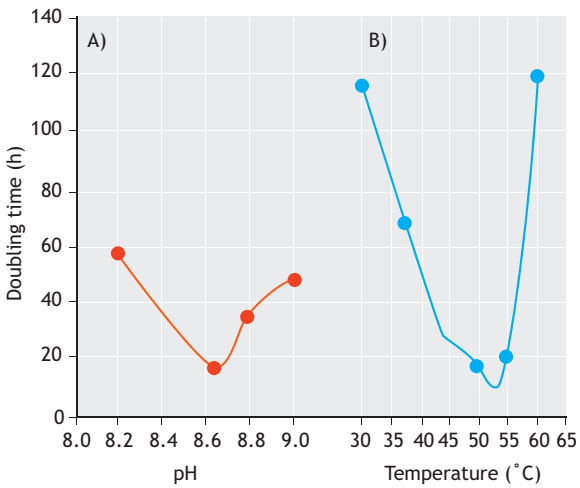

Figure 2.3.5 Influence of (A) pH and (B) temperature on the doubling time of bacterial strains capable of performing high rate sulphidogenesis (adapted from Pikuta *et al*., 2000).

Pikuta *et al*. (2000) showed the influence of pH on the doubling time of a strain (referred to as $S1^T$) capable of showing high rate sulphidogenesis. As illustrated in Figure 2.3.5, the optimum pH for growth was between 8.0 and 9.15, while the doubling times varied between 20 and 58 h depending on the initial pH values. The doubling time of another sulphate-reducing strain HHQ 20 isolated using enrichments with hydroxyhydroquinone and sediment from Venice was lowest (40 h) in the pH range of 6.9-7.2, while at pH 5.0 and 8.0 the doubling times were threefold higher (Reichenbecher and Schink, 1997).

2.3.3.5 Oxygen

SRB are known to be anaerobic organisms. However, sulphate-reduction activity has been reported in oxic regions of microbial mats and sea sediments but, to date, there is no known pure culture capable of performing dissimilatory sulphate-reduction in the presence of oxygen concentrations larger than 1 µM (Cypionka, 2000; Kjeldsen *et al*., 2004). Kjeldsen *et al*. (2004) performed short and long term oxygen exposure experiments and monitored sulphate-reduction rates under anoxic incubation conditions (Figure 2.3.6).

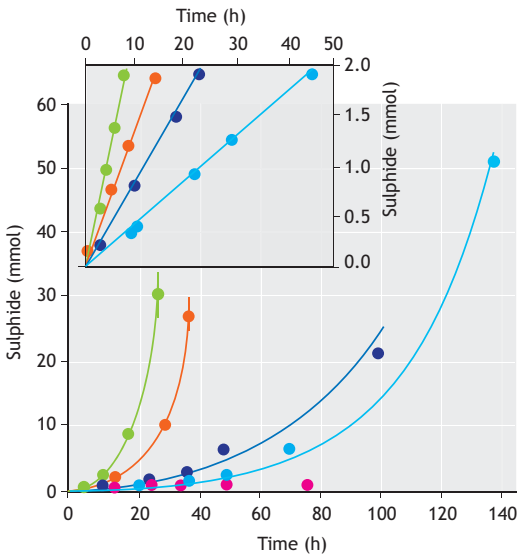

Figure 2.3.6 Sulphide production profiles in batch incubations of sludge samples exposed to 0 h (●), 33 h (●), 73 h (●), and 121 h (●) of constant aeration. The symbol (●) represents a sample that was sterilized and exposed to 121 h of aeration. The inset represents the initial linear phases of the respective curves of the same experiment (adapted from Kjeldsen *et al.*, 2004).

The sulphate-reduction rates, calculated from the initial linear increase in sulphide concentrations, decreased from 0.24 to 0.04 μM h^{-1} when the aeration time was increased from 3 to 121 h. This was attributed to temporary inactivation of an increasing fraction of the SRB population. As shown in Figure 2.3.6, the sulphide production curves follow a biphasic profile, i.e. an initial linear increase resulting from the activity of a constant number of SRB followed by an exponential increase representing the reactivation of SRB. The authors, who monitored the oxygen concentration in the bulk liquid phase and not inside the sludge flocs, raised an important question on whether the SRB were protected from oxygen exposure in anoxic micro-niches created by high respiratory activity and diffusion resistance inside the flocs during aeration experiments. The results from their experiment suggest that SRB are capable of adapting well to the situation and short exposure times to oxygen do not necessarily affect their sulphate-reduction activities.

2.3.4 Experimental setup

To estimate the sulphate-reduction rate or sulphide production rate of a specific activated sludge reactor, one activity test should be performed if the reactor is operated under steady-state conditions for at least three SRT. However, if one would like to envisage the evolution of the rate over time, after changing the bioreactor's process conditions, the rate should be checked more frequently. For instance, depending on (*i*) the research question to be addressed, (*ii*) the operating conditions, (*iii*) substrate and (*iv*) toxic compounds (if any) present, SRB activity tests should be performed in triplicates, at least once every two weeks.

2.3.4.1 Estimation of volumetric and specific rates

Table 2.3.5 gives an overview of parameters of interest. The volumetric rates of sulphate-reduction or sulphide production can be calculated from the highest slope of the exponential phase for each experiment. The equations for the volumetric and specific activity rates are:

$$r_{SO4,An} = \ln\left(\frac{S_{SO4,final}}{S_{SO4,initial}}\right) / \text{Time} \qquad \text{Eq. 2.3.3}$$

$q_{SO4,An}$ = Volumetric activity / Initial biomass concentration

or,

$$q_{SRB,SO4,An} = \frac{r_{SO4,An}}{X_{SRB}} \qquad \text{Eq. 2.3.4}$$

Where, $S_{SO4,final}$ is the final concentration of sulphate (mg SO_4^{2-} L^{-1}) and $S_{SO4,initial}$ is the initial concentration of sulphate (mg SO_4^{2-} L^{-1}) in a batch activity test, respectively.

Table 2.3.5 Stoichiometric and kinetic parameters of interest for SRB-containing activated sludge (AB: active biomass).

Parameter	Symbol	Typical units	
Stoichiometric			
SO_4^{2-} reduction to VFA consumption ratio	$Y_{SO4/VFA,An}$	S-mol C-mol^{-1}	mg SO_4^{2-} mg VFA^{-1}
Kinetic			
Specific VFA consumption rate	$q_{SRB,VFA,An}$	C-mol C-mol^{-1} h^{-1}	mg VFA mg AB^{-1} h^{-1}
Specific SO_4^{2-} uptake rate	$q_{SRB,SO4,An}$	S-mol C-mol^{-1} h^{-1}	mg SO_4^{2-} mg AB^{-1} h^{-1}

2.3.4.2 The reactor

To assess the presence and activity of SRB, batch tests should be conducted under anaerobic conditions either using airtight reactors (see Sections 2.2.2.1 and 2.2.4.1)

or serum bottles (Figure 2.3.7). The reactor(s) or serum bottles used for the execution of tests must have the required means to: (*i*) avoid oxygen intrusion under anaerobic conditions, (*ii*) provide satisfactory mixing conditions, (*iii*) maintain an adequate and desirable temperature, (*iv*) control pH, and (*v*) have additional ports for sample collection and addition of influent, solutions, gases and any other liquid medium or substrate used in the test.

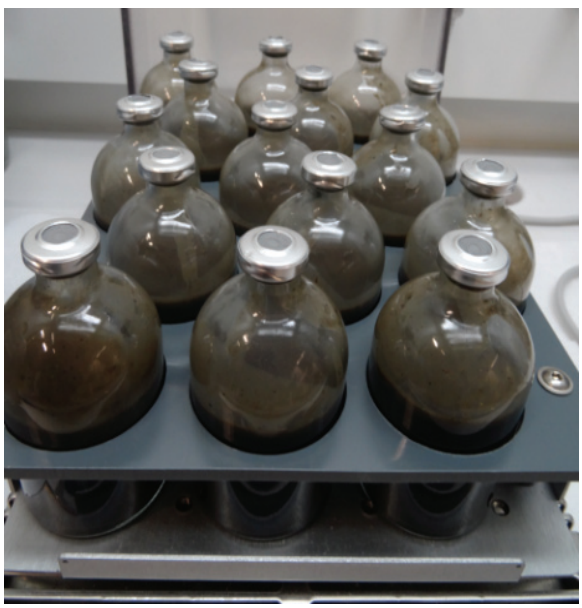

Figure 2.3.7 Batch serum bottles containing SRB sludge placed in a shaker (photo: van den Brand, 2015).

Moreover, several serum bottles can be used in parallel, as an alternative to determine the kinetic rates in batch activity tests. This practice is known as using sacrificial bottles. They are of particular interest when pure SRB cultures are to be used to avoid contamination but also to assess the kinetic conversion rates under strict anaerobic conditions and without jeopardizing the gas-liquid equilibrium phase, which can be easily altered when sampling. In this case, when using sacrificial bottles, all the batch experiments should be started with identical initial biomass concentrations that will allow the estimation of sulphate-reduction rates and sulphide production rates. In order to perform these tests and to maintain the purity of the original SRB (when required), different glass vials should be used maintaining similar experimental conditions and sacrificing one vial for each sample. By maintaining multiple vials for the same test condition, the possibility of contamination during sampling can be completely avoided and the gas and liquid phase volumes will remain constant. As explained previously, sufficient care should be taken to provide the strictly anaerobic environments required for SRB growth. Apart from measuring sulphide in the liquid phase, the gas-phase hydrogen sulphide profiles should also be monitored in order to close the sulphur balance within the test bottles.

2.3.4.3 Mixing

When airtight reactors are used, mixing conditions can be provided as described in Section 2.2.2.1 which covers EBPR. When serum bottles are used, mixing conditions are provided by means of an orbital shaker in which several batch bottles can be placed and operated at the same time. Proper mixing should be ensured and stratification, which might result due to variations in settling capacity of activated sludge, should be avoided. The shaker speed should be adjusted in such a way that no disturbance is caused to the floc structure.

2.3.4.4 pH control

pH affects the different metabolic processes of SRB, MET and AC and therefore needs to be carefully controlled and monitored. In reactors, the pH can be controlled as described in Section 2.2.2.1 on EBPR. In serum bottles, pH may be controlled by the presence of a buffering agent in the medium. This can be done by the addition of buffers such as $CaCO_3$ or phosphate buffer during the start of the incubation. Some authors have recommended the addition of sodium bicarbonate buffers or Tris buffer made from an amine base $(HOCH_2)_3CNH_2$ to control pH involving microbiological works. In bioreactors, solutions made of KOH, NaOH, NH_4OH, $Ca(OH)_2$, HCl or H_2SO_4 can be automatically added to maintain the pH (Mohan *et al*., 2005).

2.3.4.5 Temperature control

The sulphate-reduction rates of SRB and their doubling time are dependent on temperature (Hulshoff Pol *et al*., 1998; Pikuta *et al*., 2000). Therefore, it is recommended to operate the reactors with automatic temperature control, following the procedures and equipment described in Section 2.2.2.1 on EBPR. For experiments with serum bottles, often a water bath, incubator or a small room with temperature control is used. This makes it possible to control the temperature of many serum bottles simultaneously.

2.3.4.6 Sampling and dosing ports

When experiments are conducted in reactors, the requirements for the sampling and dosing ports will be the same as described in Section 2.2.2.1 on EBPR. When serum bottles are used, gas or liquid samples are taken by syringes with hypodermic needles through the rubber stoppers. If there is enough headspace pressure in the flask, no oxygen intrusion into the system will occur. Alternatively, to avoid the formation of micro-holes in the stopper or septa due to repeated piercing with the needle during sampling, a three-way plastic valve fitted to a needle can be permanently fixed to the septa.

2.3.4.7 Sample collection

For specific cases, if there is interest to measure SRB activity in the sewage, raw influent samples should be taken. In this case, most of the SRB activity in the sewerage will be linked to SRB present in the biofilm attached to the sewer conduits so that the activity of SRB measured in the raw influent in the lab may not be representative of the actual activity taking place in the sewerage. Specific protocols should be followed while collecting biofilm sewer samples (Flemming *et al*., 2000).

At pilot and full-scale treatment plants, sludge samples can be collected in different places where the SRB activity needs to be addressed: primary settling tanks, anaerobic selectors, anoxic or aerobic tanks, thickeners or anaerobic digesters. In cases where experiments are conducted with laboratory-enriched SRB cultures, it is also possible to convert the continuous bioreactor or sequencing batch reactor into a batch operation mode to measure the activities. This is often considered to be beneficial as the setup is usually adequately equipped, and often more options for controlling and sampling are present, as compared to regular batch flasks. The disadvantage of performing a batch experiment in the 'parent' reactor (as is generally applicable for all microbial cultures of interest) is the possible disturbance of the system because of (a combination of) several factors such as the withdrawal of mixed liquor due to additional sampling, the application of different operating conditions required by the batch tests, changing the substrate composition and concentration, or changing the duration of phases.

2.3.4.8 Media

When activated sludge is used as a source of SRB, real wastewater can be used as substrate, following the procedures described in Sections 2.2.2.4 and 2.2.3.3 on EBPR. The medium composition depends heavily on the objective of the test. For determining the rates of SRB under the conditions applied in the parent reactor, the medium composition should be the same as the feed applied to the parent reactor. For comparison of the rate to other conditions, it could be a consideration to use an equal standard medium for each condition. However, when the research question includes an investigation of the effect of certain compounds on the SRB activity, the compound of interest should be either added to or removed from the media. If many tests of a similar nature are to be executed, it is recommended to prepare a sufficient volume of media in order to save on preparation time. As described in Section 2.3.4.4, suitable pH buffers can be added initially to the batch incubation bottles to maintain the pH.

Depending on the objectives of the activity tests, the COD composition and concentrations, as well as the sulphate concentrations present in synthetic wastewater, can vary since they are usually the subject of investigation. Moreover, the concentrations may be adjusted proportionally for the duration of the test and the sludge concentration used. Usually, concentrations of up to 50 and 100 mg RBCOD L^{-1} are used for tests with full-scale activated sludge samples and of up to 400 mg RBCOD L^{-1} with lab-enriched cultures. Regardless of the nature of the activity test, synthetic wastewater must contain the required macro- and micro-elements (such as potassium, magnesium, calcium, iron, zinc, cobalt, among others) in sufficient amounts for SRB to avoid any metabolic limitation that may jeopardize the outcomes of the batch activity.

Postgate's medium is the most commonly used medium for the growth of SRB and to carry out SRB activity tests (Postgate, 1984). It consists of (in g L^{-1}): K_2HPO_4 (0.5), NH_4Cl (1.0), Na_2SO_4 (1.2), $FeSO_4 \cdot 7H_2O$ (0.5), COD (3.0), $MgSO_4 \cdot 7H_2O$ (2.0), yeast extract (1.0), ascorbic acid (0.1), thioglycollic acid (0.1), and Na_2SO_4 (1.0). Sodium acetate ($NaAcetate \cdot 3H_2O$) can be used as the carbon source. However, several synthetic wastewater recipes for performing SRB activity tests have also been described in the literature depending on the carbon source used (Villa-Gomez *et al*., 2011). van den Brand *et al*. (2014a) recommended the following media composition: $NaAcetate \cdot 3H_2O$ (486.2 mg L^{-1}), NaPropionate (147.1 mg L^{-1}), K_2HPO_4 (37.8 mg L^{-1}), KH_2PO_4 (14.2 mg L^{-1}), NH_4Cl (382 mg L^{-1}), $MgCl_2 \cdot 6H_2O$ (93.4 mg L^{-1}), $CaCl_2$ (58.4 mg L^{-1}), trace elements (1 mL L^{-1}), and an adequate sulphate source (for example: $MgSO_4$), resulting in a sulphate concentration of 500 mg

L^{-1}. For fermentative SRB activity tests, Villa-Gomez *et al*. (2011) used the following media composition in batch and continuous bioreactors: KH_2PO_4 (500 mg L^{-1}), NH_4Cl (200 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (2,500 mg L^{-1}), $FeSO_4 \cdot 7H_2O$ (50 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (2,500 mg L^{-1}) and lactate as the electron donor. However, it is noteworthy to remember that a combination of complex carbon sources (lactate and VFA) and sulphate can be used as long as the COD/SO_4^{2-} ratio is < 0.67. However, while carrying out these tests, blackening of the media due to sulphate-reduction and sulphide production can easily be noticed. Other examples of compounds that can be used to introduce sulphate in synthetic wastewater are seawater or sodium sulphate taking into account that the COD/SO_4^{2-} ratio is 0.67. Trace elements can be prepared according to Lau *et al*. (2006). If a test requires a particular COD/SO_4^{2-}value in order to investigate the effect of limited or excess sulphate levels, the media can be simply adjusted by adding low amounts of sulphate or more COD. When the media is to be prepared in advance, follow the recommended separation of media containing COD source and N source in order to avoid biomass growth in the prepared media. The final colour of this media is transparent. If required, the synthetic wastewater can be concentrated, sterilized in an autoclave (for 1 h at 110 °C) and used as a stock solution if several tests will be performed in a defined period of time. However, the solution must be discarded if any precipitation or loss of transparency is observed.

For experiments performed with lab-enriched cultures, it is recommended to perform the tests with the same (synthetic) media that is used for the cultivation. Alternatively and similar to the case when sludge from full-scale plants is used, the effluent from the reactor can be collected, filtered through rough pore size filters to remove larger particles, and used to prepare the required media with the desired carbon and sulphate concentrations for the execution of the activity tests.

2.3.5 Analytical procedures

Analytical tests required for the SRB activity test can be performed by following standardized and commonly applied analytical protocols as described in Section 2.2.2.5 on EBPR.

However, for estimating the activity of SRB only for a few selected parameters of interest, a modification of standard techniques/protocols is required. Therefore, a detailed description of the methods for COD and sulphide determination is given below. The analysis for sulphate is also included, as this parameter is of interest in SRB activity tests. However, for each test, it is important to realize that some compounds interfere with analytical tests. For instance, COD determination is very sensitive when chloride is present in the samples. The effect of such compounds on the analytical tests should be verified by carrying out the analysis without this compound.

Concerning sulphide concentration measurements, this could be done by making a standard sulphide concentration with a known concentration and a solution with the suspected disturbing compound. Then two tests can be performed in parallel. The first test should be carried out using the standard solution and the second test using the standard solution plus the suspected compound at concentration levels that are usually expected in the mixed liquor. If the sulphide concentration is the same in both analytical tests, the effect of the potentially disturbing compound is not significant in the tests at the applied concentrations and therefore, the analytical test should be considered as appropriate for analyses.

2.3.5.1 $COD_{organics}$ and COD_{total}

The method to measure COD has been extensively described elsewhere (APHA *et al.,* 2012), though some important additions are required. It is usually straightforward to determine the total COD of the sample or only the soluble COD fraction. To measure the soluble COD fraction, the sample should be filtered using a 0.45 µm filter. The sulphide also contributes to the COD measurements, and often this contribution (interference) is not desired. Therefore, two analyses should be performed, named hereafter as $COD_{organics}$ and COD_{total}. The COD_{total} (also referred to as the total COD concentration in a soluble state), in this particular case, also includes sulphide, while in the $COD_{organics}$ analysis, interferences due to sulphide contribution can be eliminated from the sample by applying a mathematical correction and thus the COD concentration is only associated with the content of organic matter present in the sample. As recommended by Boyles (1997), prepare a separate standard for sulphide and perform a COD test on the standard, and apply a mathematical correction to the result of COD_{total}.

Another method to completely remove sulphide from the sample is based on the precipitation based procedure of Poinapen *et al*. (2009). A few drops of 10 M NaOH solution are added to the sample, as an increase in pH will induce precipitation. The H_2S/HS^- system has a pK_{s1} of 7.05 at 25 ˚C (Bjerrum *et al*., 1985) and at a pH of 10.0 most of the sulphide present in the sample is in the form

of HS^- species with a very small proportion in the form of S^{2-} species (pK_{s2} = 12.92 at 25 °C). Neither HS^- nor S^{2-} species can escape from the sample since both compounds are very well dissociated. Subsequently, zinc sulphate ($ZnSO_4$) is added to precipitate with sulphide as zinc sulphide (ZnS), which should be separated from the sample by filtration. Finally, the measured concentrations should be corrected for the dilution with the $ZnSO_4$ solution.

2.3.5.2 Sulphate

Sulphate can be measured by various techniques, such as gravimetric, ion chromatography, methylthymol blue method and turbidimetric. These methods are described elsewhere (APHA *et al.,* 2012). The most commonly used method is a Hach Lange kit, a turbidimetric-based procedure or using a high performance liquid chromatography (HPLC) equipped with an AS9-SC Column and an ED 40 electrochemical detector (Dionex).

2.3.5.3 Sulphide

Depending on the pH, sulphide can be present in various states. Therefore, it is important to perform a sulphide test in both the liquid as well as the gas phase.

Liquid samples

When samples are taken for sulphide measurements, the procedures applied are crucial as sulphide is easily lost during sampling due to stripping or chemical reactions. It is therefore important to solubilize the volatile fraction in the liquid phase, for example using NaOH solution. For the analysis of sulphide, two main procedures can be used. The first procedure is referred to as the indirect COD method, while the second procedure is the direct methylene-blue method. Both these procedures will be described in this section. 1 g of sulphide corresponds to ~2 g COD. The sulphide concentration could therefore be expressed by its COD content.

This could be achieved by measuring the COD concentration of the sample in terms of COD_{total} and $COD_{organics}$. The COD concentration should be analysed using the standard method, according to the specific treatment steps described previously (Section 2.3.5.1). The sulphide concentration could then be calculated by subtracting the $COD_{organics}$ value from the COD_{total} value, and corrected for the COD/sulphide weight ratio. The expression to calculate the sulphide content (S_{H2S} in g L^{-1}) is:

$$S_{H2S} = (COD_{total} - COD_{organics}) / 2 \qquad \text{Eq. 2.3.5}$$

Where, 2 is the sulphide to COD ratio (*w/w*).

In the direct methylene-blue method, specific for sulphide analyses, zinc acetate is used to ensure a higher degree of sulphide fixation. Subsequently, the reagents containing dimethyl-parafenyl-diamine and iron are used to form methylene blue, resulting in a blue solution. The intensity of the colour can be analysed photometrically to quantify the sulphide concentration in the sample.

Gas-phase samples

Gas-phase samples should be collected from the batch incubation bottles using airtight glass syringes (for example: Hamilton, USA). The most commonly employed method to estimate gas-phase H_2S concentrations is using gas chromatography (GC). Li *et al*. (2013) used a GC (Agilent 6890 N, USA) fitted with a flame photometric detector (FPD) and DB-1701 capillary column (30 m × 0.32 mm × 0.25 µm, Hewlett Packard, USA) to estimate H_2S concentrations. The temperature of the oven, injection, and detector were set at 100 °C, 50 °C, and 200 °C, respectively, while nitrogen was used as the carrier gas. H_2S concentrations can also be determined by titration using a standard potassium iodide-iodate as the titrant and starch indicator (APHA *et al.,* 2012). Another quick and easy method to monitor the H_2S concentrations in wastewater treatment plants is the use of gas detector tubes (Kitagawa, Japan). H_2S concentrations can also be measured using Jerome 631-X Hydrogen Sulphide Analyser (Arizona Instruments, USA). However, in cases where it is difficult to perform the analysis immediately, Tedlar bags (Tedlar gas sampling bags, Sigma-Aldrich) should be used for collecting gas samples from continuously operated reactors.

Temperature and pressure are two important factors that need to be considered during the calibration of GC for gas-phase H_2S measurements. If S^{2-} in liquid phase is added to a closed system, the resulting gas-phase H_2S concentration (C_{H2S}) in the system can be calculated as follows:

$$C_{H2S} = 22.4 + 10^6 \cdot \frac{\rho \cdot V_L}{MW \cdot V} + \frac{T}{273} + \frac{760}{P} \qquad \text{Eq. 2.3.6}$$

Where, ρ is liquid density (g mL^{-1}), V_L is the volume of liquid (mL), T is the temperature (K), P is the pressure (torr), MW is the molecular weight (g mol^{-1}) and V is the volume of the closed system (L).

2.3.6 SRB batch activity tests: preparation

This section describes not only the different steps but also the apparatus characteristics and materials needed for the execution of the SRB batch activity tests.

2.3.6.1 Apparatus

If the experiments are conducted using a reactor, the description given in Section 2.2.3 on EBPR is applicable. If the tests are conducted in serum bottles, the following apparatus is required:

1. Serum vials, including a rubber stop and aluminium crimps to secure an anaerobic environment.
2. A nitrogen gas supply.
3. A pH electrode.
4. A 2-way pH controller to add either HCl and/or NaOH (alternatively, a one-way control for HCl addition or manual pH control can be applied through the manual addition of HCl and/or NaOH).
5. A thermometer (recommendable temperature working range of 0 to 40 ˚C). Confirm that the electrodes or meters (pH, thermometer) are calibrated less than 24 h before execution of the batch activity tests in accordance with guidelines and recommendations from suppliers.
6. A room or incubator to control the temperature at the desired temperature.
7. A shaker in which the rpm can be set up to 300 rpm.
8. A pipette and tips to measure the exact volume of the samples taken.
9. Syringes and needles to take the sample from the serum vials by making a vacuum.
10. Filters with a pore size of 0.45 µm for sample filtering and for preservation purpose.
11. Additionally, all the materials required for analysis. These are elaborated in Section 2.3.5.

2.3.6.2 Materials

1. Two graded cylinders of 1 or 2 L volume (depending upon the sludge volume used) to hold the sample and wash it, if required.
2. At least 2 plastic syringes (preferably of 20 mL or at least of 10 mL volume) for the collection and determination of soluble compounds (after filtration).
3. At least 3 plastic syringes (preferably of 20 mL volume) for the collection of solids, particulate or intracellular compounds (without filtration).
4. At least 3 glass syringes (preferably of 5 mL volume) for the collection of gas-phase samples.
5. 0.45 µm pore size filters. Preferably not of cellulose acetate because they may release some traces of cellulose or acetate into the collected water samples. Consider having at least twice as many filters as the number of samples that need to be filtered for the determination of soluble compounds.
6. Transparent plastic cups with a volume of 10 or 20 mL to collect the samples for the determination of soluble compounds (e.g. soluble COD, acetate, propionate, sulphate, and sulphide).
7. Transparent plastic cups with a volume of 10 or 20 mL to collect the samples for the determination of MLSS and MLVSS. Consider the collection of these samples in triplicates due to the variability of the analytical technique.
8. A plastic box or dry ice box filled with ice with the required volume to temporarily store (for up to 1-2 h after the conclusion of the batch activity test) the plastic cups and plastic tubes for centrifugation after the collection of the samples.
9. Plastic gloves and safety glasses.
10. Pasteur or plastic pipettes for HCl and/or NaOH addition (when the pH is controlled manually).
11. Metallic lab clips or clamps to close the tubing used as a sampling port when samples are not collected from the reactor/fermenter.

2.3.6.3 Media

- **Real wastewater**
  See Section 2.2.3.3 on EBPR.

- **Synthetic media or substrate**
  If tests require synthetic wastewater, depending on the type of the tests, the synthetic influent media could contain a mixture of carbon and sulphate sources plus relevant (macro- and micro-) nutrients. Generally, they can be mixed all together in the same media or split in two solutions (*i*) COD source, and (*ii*) SO_4^{2-} and N source plus the nutrient solution).
  a. COD source solution: It must be composed of RBCOD, preferably of VFA such as acetate or propionate, depending on the nature or goal of the test and the corresponding test objective (research questions). For anaerobic batch activity tests, the concentration needs to be adjusted to ensure that the COD is consumed within the duration of the test. For batch activity tests performed with activated sludge from a full-scale plant, usually COD concentrations not greater than 100 mg L^{-1} are recommended. For lab-scale mixed liquor sludge samples, the COD concentrations can be

as high as the influent COD concentration of the lab-scale system (and even sometimes 2 to 3 times higher). An example of a COD solution is NaAcetate·$3H_2O$ (486.2 mg L^{-1}) or Na-Propionate (147.1 mg L^{-1}).

b. Sulphate source solution: Sulphate can be added as sodium sulphate. However, in some cases and especially in the case of saline wastewater treatment, sulphate is accompanied by salinity. To mimic those conditions, it is also possible to add sea salt (containing sulphate). The sulphate concentrations can be adjusted, as desired, depending on the purpose of the experiment. The nutrient solution should contain all the essential macro- (ammonium, magnesium, calcium, potassium) and micro-nutrients (iron, boron, copper, manganese, molybdate, zinc, iodine, cobalt) to ensure that SRB metabolism is not limited. This can lead to the wrong interpretation of results and in extreme cases, a near complete failure of the test. Thus, one must make sure that all the required compounds are added to the solution and in the correct amounts. The media composition is recommended in Section 2.3.4.8.

c. Washing media: If the sludge sample must be washed to remove the presence of an undesirable compound (which may be even inhibitory or toxic), prepare a fresh nutrient solution to wash the sludge containing the medium composition described above, without VFA. The washing process can be repeated two or three times following a similar procedure likewise in Section 2.2.3.5. Thereafter, the necessary preparation steps of the batch activity tests can be performed.

d. Preparing acid and base solutions: see Section 2.2.3.3 on EBPR.

If analyses are not outsourced to specialized analytical labs, the required stock and working solutions to carry out the determination of the analytical parameters of interest must be also prepared in accordance with standard methods (APHA *et al.,* 2012) and the corresponding protocols.

2.3.6.4 Material preparation

- For information on the number of samples, see Section 2.2.3.4 on EBPR batch activity tests.
- Frequency of sample collection:
  a. If the maximum specific kinetic rates must be determined (e.g. maximum specific acetate or propionate uptake rate or maximum sulphate-reduction rate), then increase the frequency of sampling during the initial few hours or days of incubation. In general, samples should be collected once every 5 min during the first 30-40 min of duration of the batch activity test, similar to the method described for EBPR tests.
  b. To ascertain the stoichiometric ratios and not the kinetic ones (e.g. anaerobic SO_4^{2-} reduction/HAc uptake ratio), then the samples can be collected only during the beginning and at the end of each phase to determine the conversions of interest within the selected period (phase).
- To increase the reliability of data collected and to determine the initial conditions of the sludge, it is strongly recommended to collect a series of samples before any media is added. Carefully define the maximum and minimum working volumes of the reactor, as described in Section 2.2.2 on EBPR.
- Preparation of sample cups can be performed as described in Section 2.2.3.2 on EBPR. A simple working plan created in a spreadsheet (Section 2.3.9) can be rather useful to perform and keep track of the sample collection frequencies. Furthermore, this spreadsheet can be used to maintain a database of the different batch tests carried out. Organize all the required material within a relatively close radius of action, around the batch setup, so that delay in handling and preparing the samples can be avoided. Calibrate all the meters (pH and thermometer) less than 24 h prior to the execution of the tests and store them in proper solutions until the execution of the tests, following the particular recommendations of the corresponding manufacturer or supplier and confirm that their readings are reliable before the start of the tests. Samples must be properly stored and preserved until they are analysed (Table 2.3.6).

Table 2.3.6 Recommended sample storage and preservation procedure for the determination of SRB activity tests.

Parameter	Material of sample container	Method of preservation	Maximum recommended time between sampling, preservation procedure and analyses
Sulphate	Plastic or glass	Filter immediately after sample collection thorough 0.45 µm pore-sized filter and cool to 1-5 °C, or freeze to -20 °C.	24 h for samples stored at 1-5 °C; up to 1 month for frozen samples.
Sulphide	Plastic or glass	Immediately solubilize in a drop of 1M NaOH.	Recommended to perform these measurements as quickly as possible.

2.3.6.5 Mixed liquor preparation

These procedures consider that batch activity tests can be performed as soon as possible after collection of samples from full- or lab-scale systems or, in the worst case scenario, within 24 h after collection. Performing the batch tests 24 h after the collection of mixed liquor sludge samples is not recommended because the SRB culture can undergo potential biochemical changes during handling (unless the exposure time after collection is of particular interest for performing these tests). Bearing in mind the previous comments, the following three protocols are recommended to prepare the mixed liquor samples for conducting batch activity tests:

1. If batch activity tests can be performed in less than 1 h after collection of the sludge sample and if the sludge sample does not need to be washed:
   a. Adjust the temperature of the batch reactor where the tests will take place to the target temperature of the study.
   b. Collect the sample at a wastewater treatment plant (WWTP) from one of the following:
      - From the raw influent.
      - At the end of the aerobic tank or stage.
      - At the outlet of the primary or secondary sludge thickeners or anaerobic digesters.
      - Take sludge from a laboratory reactor.
   c. Transfer the sludge sample to the reactor or serum bottles where the batch activity tests will take place.
   d. Start a gentle mixing (50-100 rpm in the case of the reactor) and follow up the temperature of the sludge sample by placing a thermometer inside the reactor or serum bottle (if the setup does not have a built-in thermometer).
   e. Maintain mixing conditions until the sludge has reached the target temperature of the study.
   f. Maintain the anaerobic conditions by using an airtight reactor and sparge N_2 gas to avoid/reduce oxygen intrusion.
   g. If nitrate is detected in the aerobic samples collected to perform these tests then follow instructions presented in Section 2.2.2.3 on EBPR. Alternatively, for nitrate concentrations greater than 10 mg NO_3^--N L^{-1}, 9 mg COD could be added per every mg of nitrate detected (9 mg COD mg NO_3^--N^{-1}). As soon as nitrate is no longer observed, the sludge can be immediately used to conduct the anaerobic batch activity tests. Nitrate or nitrite detection strips (Sigma-Aldrich) can be used for a quick estimation of the presence of these compounds.
2. If batch activity tests can be performed in less than 1 h after collection of the sludge sample but the mixed liquor sludge sample needs to be washed, see washing procedures described in Section 2.2.3.5.
   a. Transfer the 'washed' sludge sample to the reactor or fermenter where the batch activity tests will be performed.
   b. Start a gentle mixing (50-100 rpm in the case of the reactor) and follow up the temperature of the sludge sample by placing a thermometer inside the reactor (if the set-up does not have a built-in thermometer).
   c. Maintain mixing conditions until the sludge has been exposed to the target temperature of study for at least 30 min.
3. In some cases, due to location and distance issues, the tests cannot be performed in less than 1 or 2 h after collection (but within 24 h):
   a. Adjust the temperature of the batch reactor to the target temperature of the study.
   b. Keep the sludge sample cold until the commencement of the test (e.g. by placing the bucket or jerry can in a refrigerator at 4 °C).
   c. Prior to the test, take out the sludge sample from the refrigerator, cool box or cold room.
   d. Mix the contents gently in order to obtain a homogenous and representative sample with a similar MLSS concentration as in the original lab- or scale system from where it was originally collected.
   e. If the sample needs to be washed, wash the sludge in a mineral solution (see Section 2.3.6.3 on working solutions).
   f. Transfer the washed sludge to the reactor or fermenter where the batch activity test will take place.
   g. Start to flush N_2 gas in the sludge sample while mixing gently to remove any dissolved oxygen.
   h. Follow the temperature of the sludge sample by placing a thermometer inside the reactor (if the setup does not have a built-in thermometer).
   i. Keep flushing N_2 gas and mix the contents for at least 1 h (maximum 2 h), but ensure that the sludge is exposed to the target temperature of the study for at least 30 min.
   j. After the procedure of adjusting the temperature, if nitrate is detected (see Section 2.3.6.3), add a solution containing readily biodegradable COD at a 9 mg COD mg NO_3^--N^{-1} ratio. As soon as nitrate is no longer observed, the sludge can be immediately used to start and conduct the anaerobic batch activity tests.

2.3.6.6 Sample collection and treatment

For the purpose of this activity test, three types of samples are required: *(i)* a filtered sample, *(ii)* a filtered sample with NaOH and *(iii)* a biomass sample. Table 2.3.7 provides a list of analyses corresponding to different sample collection and treatment steps.

Table 2.3.7 List of analyses performed on each sample.

Sample	Parameter
Filtered sample	COD_{total}; $COD_{organics}$; Sulphate
Filtered sample with NaOH	Sulphide
Biomass sample	MLVSS; MLSS

- **Filtered sample**

The filtered sample is used for the analyses of COD_{total}, $COD_{organics}$ and sulphate. A well-mixed sample is taken from the serum vial with a needle and a syringe. The needle is injected into the rubber stopper. Then the serum bottle is turned upside down, so that the needle is immersed in the liquid phase. The syringe is pulled in order to create a vacuum and collect the sample. Subsequently, the sample is directly filtered using a 0.45 µm filter, to avoid further conversions. Thereafter, the COD_{total}, $COD_{organics}$ and sulphate concentration are measured according the protocol described in Section 2.3.5.

- **Filtered sample with NaOH**

The filtered sample in which a few drops of NaOH are added is used for the analyses of the sulphide concentration. The sample should be transferred into a tube containing three drops of 1 M NaOH directly after sampling. This is done by using a needle and a syringe. This needle is pierced into the rubber stopper (septa). Then the serum bottle is turned upside down, such that the needle is immersed in the liquid phase. The syringe is pulled back in order to create a vacuum and collect the sample. The sooner the sample is mixed with NaOH, the less sulphide is lost during sampling. Thereafter, the sample is filtered using a 0.45 µm pore-size filter and analysed as described in Section 2.3.5. This analysis should be performed directly after collecting the sample, to avoid sulphide losses.

- **Gas sample**

Gas-phase samples should be collected from the batch incubation bottles using air-tight glass syringes. A calibration plot should be prepared in the GC using standard H_2S gas cylinders (example: 25 to 1,000 ppm). Gas-phase analysis should also be performed immediately after collecting the sample from the glass bottles in order to avoid losses.

- **Biomass sample**

In order to estimate MLVSS and MLSS concentrations, a well-mixed sample from the reactor should be collected. The easiest method is to use a syringe and needle arrangement. Ensure that you know the exact volume taken from the reactor. The MLVSS and MLSS should be analysed in triplicate as described elsewhere (APHA *et al.,* 2012). The frequency of analysing COD, sulphate and sulphide depends on the biomass activity. After data processing, a linear line (as presented in Figure 2.3.8) is required for proper analyses. The more data points present on this linear line, the better, but a balance between investments (in money and time) suggests that four points on the linear line are already sufficient. The safest option is to take several samples during the first hour, once every 10 or 15 min. The best way to deal with this is to perform extensive sampling in the first tests (as a trial), and based on these results, a design can be made to ascertain how much data points are actually required to perform subsequent tests.

2.3.7 Batch activity tests: execution

This section describes how a batch activity test of SRB should be executed. The description consists of a material and chemical list, protocol to prepare media, sampling scheme (time frame), sample collection, and analysis. This section concludes with a step-by-step approach to performing sulphate-reducing bacteria activity tests. The material and chemical list specific for the analytical tests, as well as how the tests should be conducted, is described in Section 2.3.6.

The general advice as provided in Section 2.2.4 on EBPR should be following accordingly for the SRB activity tests. Table 2.3.8 gives examples of typical tests performed to ascertain SRB activities.

Table 2.3.8 Typical tests for SRB activity analyses.

Test code	Redox	Short description and purpose
SRB.ANA.1	Anaerobic	Performed with real wastewater, for instance, the raw influent, to determine the exact rate of SRB under that particular condition.
SRB.ANA.2	Anaerobic	Conducted using a defined standard media (see Section 2.3.4.8), to compare the sulphate-reduction rate from SRB under different conditions.

Test SRB.ANA.1 Anaerobic SRB activity test

The first step of conducting a SRB activity test is to ensure the correct composition of medium and following a correctly designed experimental schedule (Section 2.3.8.2). Both steps heavily rely on the goal and scope of one's research, but also on the origin of the SRB sludge (enriched culture, mixed culture or culture collected from a full-scale or lab-scale system). The required volume of the sludge sample depends on the objective of the study and the research questions. It should be ensured that the biomass and liquid/gas phase concentrations are comparable as much as possible to the original situation, unless required otherwise. After the experimental design, the steps shown below should be followed. The same approach is also valid for SRB activity tests in reactors.

1. Collect the items on the material and chemical lists.
2. Prepare the required stock solutions for conducting analytical tests.
3. Prepare the media stock solutions.
4. Fill the serum vials with media and correct the pH, if necessary, with HCl and/or NaOH.
5. Transfer the SRB sludge into the serum bottles (preserving the anaerobic conditions as much as possible, for example, by avoiding turbulence).
6. Close the serum bottles with a rubber stopper and aluminium crimp.
7. Flush both the headspace and the liquid phase in the serum bottles with N_2 gas. This is done by injecting a needle with the gas supply and placing another needle slightly above the liquid phase from which the overpressure can escape. This latter needle should not touch the liquid phase; otherwise, the sludge will start to escape. For 80-110 mL bottles, 1 min flushing is enough to obtain the required anaerobic conditions, but this could be checked by the addition of resazurin (dying colour). The media becomes colourless after becoming anaerobic. Remove both needles from the septa at exactly the same time. The bottles should now be anaerobic.
8. Now take a sample at time t = 0 and handle this and other samples immediately. The sample is collected using a syringe. Then turn the bottle such that the needle touches the liquid (avoid taking out biomass). Pull the syringe back to collect the sample.
9. Collect the sulphide sample in 1 drop of 1 M NaOH, and the rest can be used for COD and sulphate analyses. Sulphide samples should be solubilized in NaOH and immediately analysed.
10. Follow the time scheme of the sampling.
11. After finishing the time scheme, open the serum bottles and collect the biomass sample and perform MLVSS and MLSS analyses.
12. If the experiments cannot afford SRB sludge losses and it is desired to put the sludge back into the parent reactor, wash it first three times with the media used to feed the parent reactor. If the media composition is not known, use demineralised water for washing.
13. Then the data should be analysed using the procedure described in Section 2.3.8.
14. Note: if the reactor is used for batch activity tests, a similar procedure as described for EBPR anaerobic tests should be followed (see Section 2.2.4).

Test SRB.ANA.2 Anaerobic SRB activity test

A similar approach should be followed for SRB.ANA.2 using synthetic media (Section 2.3.6.3) to estimate the SRB activity.

2.3.8 Data analysis

2.3.8.1 Mass balances and calculations

The experimental data can be used to calculate the sulphate-reducing activity of SRB (Section 2.3.8.2). Sulphide formation is a result of SRB activity and the variability of rate coefficients depends on the carbon source, initial sulphate concentrations and the presence of specific SRB genera. The additional COD, sulphate and sulphide concentration measurements taken at time t = 0 and at the end of the measurement (effluent) should be used to check the mass balances. Rate determination can only be reliable with the correct mass balances in place. The mass balance equations for COD and sulphur compounds can be represented as follows:

COD balance

$$COD_{organics,in} + S_{H2S,in} = COD_{organic,out} + S_{H2S,out} \quad \text{Eq. 2.3.6}$$

Sulphur balance

$$S_{SO4,in} + S_{H2S,in} = S_{SO4,out} + S_{H2S,out} \quad \text{Eq. 2.3.7}$$

Also it is possible to calculate an electron balance.

To calculate the biological sulphate-reduction rate (only when the mass balances are correct), the following calculations should be executed:

a. Use a standard calibration curve to convert the measured optical density values (OD_{675}) from the spectrophotometer to concentration units (mg L^{-1}).

b. Convert the sulphide concentration from mg L^{-1} into mM (if desired). The molar mass of completely dissolved sulphide (S^{2-}) is 34 g mol^{-1}.
c. Take the average value of the triplicate.
d. To obtain a profile indicating the amount of sulphide produced, correct for the sulphide concentration present at time t = 0 min (by subtracting this value from all the values).
e. Determine the slope from the first samples, in which a linear line is expected.
f. Divide the slope value by the amount of biomass present (g VSS), to determine the rate in mmol SO_4^{2-} g VSS^{-1} h^{-1}). The formation of 1 mol sulphide is related to the removal of 1 mol sulphate. Also a correction from molarity to mol is required.

2.3.8.2 Data discussion and interpretation

The results of COD, sulphate and sulphide analyses of the samples taken during the start and the end of the test can be used to check the mass balances. As sulphide is easily lost during sampling, a mass balance of 100 % is not easy to obtain. Therefore, a mass balance fit of 95 % is considered to be satisfactory in order to continue data analyses. If only sulphide formation in a batch test is analysed to measure the biological sulphate-reduction rate by SRB, it is important to perform the test at least in triplicate. It is also very important to check the mass balances, which is possible by executing COD and sulphate analyses at time t = 0 and at the end of the test. The methylene-blue method is a very sensitive and reliable technique. Therefore, when the mass balance fit is around 95 %, the sulphide accumulation profile can be the basis for the calculation of the reduction rate. It is also possible from this type of analyses to determine the characteristic conversion parameters, such as the maximal conversion rate. To measure the maximal conversion rate, it is crucial that all compounds are present in excess. Then, the maximal conversion rate can be determined as described in Sections 2.3.9 and 2.2.5.2. The Lineweaver-Burkplot method can be used to determine the characteristic parameters. This method is described in detail by Nelson and Cox (2005).

There are only limited results available for an SRB activity test, specifically developed for domestic wastewater treatment. Some available literature results are presented in Table 2.3.9 and evidently a wide range of sulphate-reduction rates have been reported depending on the operating conditions that facilitated SRB activity. Although the SRB are strictly anaerobic microbes, some studies (e.g. Lens *et al.*, 1995) are based on aerobic wastewater treatment, and sometimes with low sulphate concentration in the influent, resulting in rates that were significantly lower than the ones observed by van den Brand (2014a; 2014b; 2015).

Table 2.3.9 Sulphate-reduction rates ($q_{SRB, SO4,An}$) reported in the literature from sludge taken from reactors after long-term operation.

System	Scale	$q_{SRB,SO4,An}$ (mg SO_4^{2-} g VSS^{-1} h^{-1})	References
RBC	Full	1-11	Lens *et al.* (1995)
CAS	Full	0-3.1	Lens *et al.* (1995)
CAS	Laboratory	1.9	Lens *et al.* (1995)
ANS	Full	6-7.3	Lens *et al.* (1995)
ANS	Laboratory	11.6	Lens *et al.* (1995)
ANS	Laboratory	100-220	van den Brand *et al.* (2014a; 2014b; 2015)

RBC: Rotating Biological Contactor
CAS: Conventional Activated Sludge
ANS: Anaerobic Sludge

2.3.9 Example

Table 2.3.10 shows an example of worksheet that can be used for different SRB activity tests. The example concerns the tests solely based on the production of sulphide and COD, sulphate and VSS measurements taken during the beginning and end of the experiments. Figure 2.3.8 is an example of a test performed with synthetic wastewater that also includes the analysis of VFA (acetate and propionate).

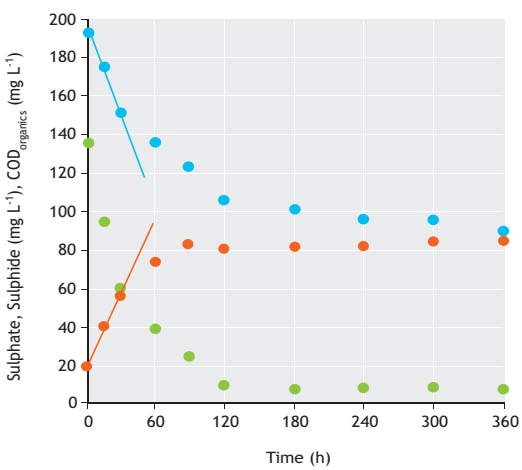

Figure 2.3.8 Example of a graph depicting sulphide (●), sulphate (●) and $COD_{organics}$ (●) concentrations during the SRB.ANA.2 activity test from which the sulphate-reduction rate can be calculated.

For this test, it is suggested to perform several (at least 10) COD and sulphate measurements, and VSS/TSS measurements from the sludge at the end of the test, as described in Section 2.3.7.

Using the protocol described in Section 2.3.5, a graph is obtained, from which the slope can be determined. A typical profile obtained from an SRB activity test is presented in Figure 2.3.8. The slope calculated in this example is 225 mg SO_4^{2-} h^{-1}, which corresponds to 2.34 mmol SO_4^{2-} h^{-1}. The amount of biomass in the reaction vessel was 4.5 g MLVSS. The working volume of the batch reactor was 2.5 L. The actual biological sulphate-reduction rate was subsequently calculated as follows:

$$q_{SRB,SO4,An} = \frac{2.34}{4.5} = 0.53 \text{ mol } SO_4 \text{ g VSS}^{-1} \text{ h}^{-1} \qquad \text{Eq.2.3.8}$$

SRB activity tests could also be used to investigate the effect of compounds on the sulphate-reduction rate. Figure 2.3.9 presents an example of a study comparing different nitrogen compounds (ammonium, nitrate and nitrite) at different N concentrations as performed by van den Brand *et al.* (2015). By calculating the sulphate-reduction rate under specific conditions as performed in Figure 2.3.9, and by presenting all the SRB rate results in one figure, the effect of nitrogen compounds on the sulphate-reduction rate can be analysed. In this case error bars are included to show the accuracy of the measurements. The sulphate-reduction rate decreased in time with an increased nitrogen concentration (Figure 2.3.9).

Table 2.3.10 A typical example of a worksheet for an SRB activity test (type SRB.ANA.2). Note that when more information regarding the kinetics is desired, sulphate and $COD_{organics}$ analysis should be performed during all the selected times. However, instead of $COD_{organics}$, the actual acetate and propionate concentrations should be measured.

Anaerobic sulphate reduction batch tests — **Code: SRB.ANA.2**

Date:	Wednesday, 09.10.2015 10:00 h
Description:	Test at 10 °C, pH 7, synthetic substrate and enriched SRB culture
Test No.:	1 of 3
Duration	6.0 h (360 min)
Substrate:	Synthetic: acetate and propionate (total 400 mg L^{-1})
Sampling point:	Serum vial using needle
Samples No.:	SRB.ANA.2(1.1-1.11)
Total sample volume:	80 mL
	(20 mL for MLVSS, 6 mL for other samples)
Reactor volume:	2.5 L source sludge, 80 mL serum vial

Experimental procedure in short:

1. Confirm availability of sampling material and required equipment.
2. Confirm calibration and functionality of systems, meters and sensors.
3. Fill serum vial with media and correct for pH.
4. Take sample for COD (acetate and propionate: $COD_{organics}$) in the feed.
5. Transfer 100 mL of sludge to serum vial.
6. Close serum vial with a rubber stop and aluminium cap.
7. Flush serum vial headspace with N_2 gas.
8. Place vial on mixing plate and take sample at t = 0.
9. Take other samples following the sampling scheme below.
10. After last sample is taken, measure biomass concentration of the remaining vial content.
11. Verify that all systems are swtiched off.

Sampling schedule

Time (min)	-20	0	15	30	60	90	120	180	240	300	360
Time (h)	-0.33	0.00	0.25	0.50	1.00	1.50	2.00	3.00	4.00	5.00	6.00
Sample No.	1	2	3	4	5	6	7	8	9	10	11
Parameter	ANAEROBIC PHASE										
COD (mg L^{-1})	396[1]	136	95	59	39	25	9	8	8	8	8
Sulphide (mg L^{-1})		18	40	58	74	83	81	83	83	84	85
Sulphate (mg L^{-1})		195	176	153	137	124	107	102	96	96	91
MLSS and MLVSS (mg L^{-1})											See table

[1] Average value of the COD concentration present in the synthetic substrate prior the start of the test

MLSS and MLVSS measurements

Sampling point	Cup No.	W1	W2	W3	W2-W1	W2-W3	MLSS	MLVSS	Ratio
End anaerobic phase	1	0.08835	0.10741	0.08849	0.01906	0.01892	1,906	1,892	0.99
	2	0.08835	0.10759	0.09018	0.01924	0.01742	1,924	1,742	0.91
	3	0.08834	0.10683	0.08940	0.01849	0.01742	1,849	1,742	0.94
						Average	1,893	1,792	0.95

Biomass composition

Sampling point	End anaerobic phase
MLSS (mg L^{-1})	1893
MLVSS (mg L^{-1})	1792
Ratio	0.95
Ash (mg L^{-1})	101

Note:

Acetate (CH_2O)	30.03	mg C-mmol^{-1}
Propionate ($CH_2O_{0.67}$)	24.69	mg C-mmol^{-1}
Sulphide (H_2S)	34.08	mg S-mmol^{-1}
Sulphate (SO_4^{2-})	96.06	mg S-mmol^{-1}

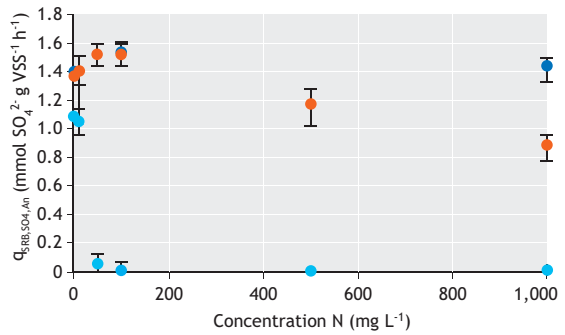

Figure 2.3.9 Example of a graph that shows the biological sulphate-reduction rate ($q_{SRB,SO4,An}$) in the presence of ammonium (●), nitrate (●) or nitrite (●) as N source in the feed, respectively.

It is also possible to study the relation between the sulphide concentration and the actual sulphate-reduction rate. Therefore, it is important to determine the moving average along the profile, in order to know the rate for the actual average sulphide concentration present in the sample. The moving average is a result of the average of three rates. These rates correspond to the sulphide concentration at which the moving average is calculated, and one rate at higher and lower concentrations. Figure 2.3.10 is an example of a typical relation between the sulphate-reduction rate and the actual sulphide concentration, based on the moving average method. This type of graph can also be prepared for the actual sulphate or COD concentration.

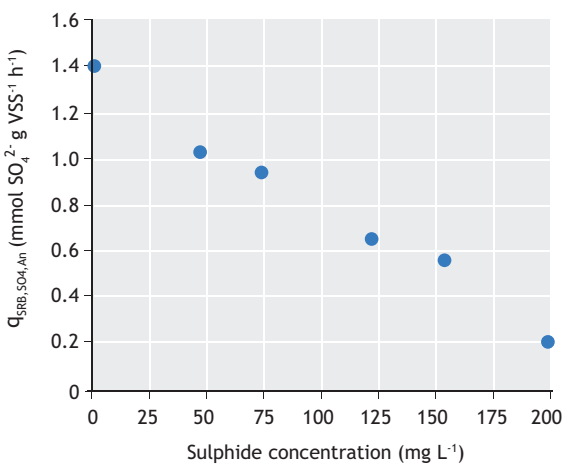

Figure 2.3.10 The relation between the sulphate-reduction rate ($q_{SRB,SO4,An}$) and the actual sulphide concentration, calculated by moving average method.

2.3.10 Practical recommendations

Volumetric sulphate-reduction rates and SRB activities can be estimated by performing experiments under controlled laboratory conditions. Figure 2.3.11 is an example of a suspended growth bioreactor, fitted with adequate monitoring devices for pH and temperature control. Anew, such reactor configurations will facilitate the ease of collecting both liquid and gas phase samples. If an aerobic test is conducted to ascertain the influence of oxygen on SRB activity, then the same tests should be carried out, except for headspace flushing with N_2-gas. If a toxicity test is desired, then the tests using synthetic wastewater should be performed and the toxic parameter added to the experimental design. The test should be repeated by using the same sludge that was exposed to that toxic compound as the inoculum to investigate the ability of the sludge to recover from toxic stress. From a microbial perspective, if one wishes to determine the time-dependent development of the SRB community structure within the sludge, molecular biology tools like fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S ribosomal DNA (rDNA) can be used (see Chapter 8).

Figure 2.3.11 Collection of a liquid sample to determine the activity of sulphate-reducing bacteria (SRB). Note the characteristic black colour of the enriched SRB biomass in the reactor (photo: KWR Watercycle Research Institute, 2014).

2.4 BIOLOGICAL NITROGEN REMOVAL

2.4.1 Process description

The need to remove nitrogen from wastewaters arises from its potential toxic effect on aquatic life in receiving water bodies as free ammonia (NH_3), its effect on the nitrogenous oxygen demand and on its role as a nutrient in enhancing eutrophication especially in marine environments (Metcalf and Eddy, 2003). Nitrogen is mainly present in wastewaters in its reduced form as ammonium (NH_4) and can be removed by different physicochemical and biological processes. The selection of the best alternative is commonly based on cost effectiveness. In general, physicochemical methods such as ammonium air-stripping, breakpoint chlorination and selective ion exchange are characterized by higher operational costs that are considered economically feasible only when the ammonium concentrations are higher than 5 g N L^{-1} (Mulder, 2003). In wastewater streams containing less than 5 g N L^{-1}, as in several industrial effluents and most municipal wastewaters (where typical nitrogen concentrations are usually lower than 100 mg N L^{-1}), biological nitrogen removal processes are usually preferred due to lower operational costs. Different biological processes, and combinations thereof, can be applied involving the relative metabolic pathways. Among the resulting alternatives, the wastewater characteristics in terms of the influent COD/N ratio will provide guidance in determining the most suitable biological processes for nitrogen removal. Arguably, three influent COD/N ratio ranges can be distinguished:

(i) For high influent COD/N ratios (> 20 g COD g N^{-1}), the nitrogen requirements or nitrogen assimilation of heterotrophic bacteria (ordinary heterotrophic organisms: X_{OHO}) for biomass synthesis during COD (organic matter) removal is usually sufficient to achieve the required nitrogen concentration in the effluent.

(ii) For influent COD/N ratios comprised between 5 and 20 g COD g N^{-1}, the combination of nitrogen assimilation for microbial growth and the application of conventional nitrification and heterotrophic denitrification processes can be applied.

(iii) For COD/N ratios lower than 5 g COD g N^{-1}, conventional nitrification and heterotrophic denitrification processes can hardly reach satisfactory nitrogen removal levels. In particular, the heterotrophic denitrification process will be limited by the lack of organic matter and an additional carbon source needs to be externally dosed. Thus, non-conventional nitrogen removal processes performing via the so-called 'nitrite-route' are more suitable for nitrogen removal because of their lower (or even absent) COD requirements. For this reason, besides other technical and economic considerations, processes such as partial nitritation-denitritation (also known as nitrite shunt) or partial nitrification-anammox (PNA) are nowadays state-of-the-art biological nitrogen removal processes for wastewaters with low influent COD/N ratio. Due to the relatively lower growth rate of anaerobic ammonium oxidizing bacteria, the anammox (anaerobic ammonium oxidation) process is currently mainly applied for the treatment of warm streams (> 25 °C) such as the supernatant from the anaerobic sludge digestion systems in municipal and industrial WWTPs.

Overall, depending on the wastewater characteristics and local conditions, the removal of nitrogen from wastewater can be performed by several technologies and combinations thereof. In spite of the different technologies and N-removal processes, in practically all of them ammonium is first (fully or partially) oxidized to nitrite (nitritation) or to nitrate (nitrification) and then the oxidized form of nitrogen is reduced to dinitrogen gas which is released into the atmosphere via either denitrification (from nitrate to N_2), denitritation (from nitrite to N_2) or anammox (from nitrite and ammonium to N_2) processes.

The nitrogen removal efficiency of the biological processes depends on an adequate balance between the activities of the different microbial groups. In this regard, the execution of batch activity tests to assess and determine the stoichiometry and kinetic rates of these biological conversion processes represents a useful tool for monitoring and controlling the nitrogen removal processes. Before describing in detail the batch test methodologies and procedures, the biological processes of nitrification, denitrification and anaerobic ammonium oxidation are briefly presented.

For a deeper understanding of different process configurations, operational conditions and factors affecting each process as well as the metabolism involved in the biological nitrogen removal cycle, the reader is referred to standard textbooks (e.g. Henze *et al.,* 2008; Grady *et al.,* 2011).

2.4.1.1 Nitrification

Nitrification is the name given to the production of nitrate by Schlœsing and Müntz (1877) who first recognized the biological nature of the process. One decade later, Winogradsky (1890) isolated for the first time an ammonium-oxidizing bacterium showing that specific groups of bacteria were responsible for nitrification. The immense work of bacterial cultivation performed by Winogradsky (1892) led to the isolation of *Nitrosomonas europea* and *Nitrobacter,* showing that the production of nitrate from the oxidation of ammonium was actually divided into two distinct microbiological processes performed by two phylogenetically independent groups of chemolithoautotrophic aerobic bacteria: ammonium-oxidizing organisms (X_{AOO}) producing nitrite and nitrite-oxidizing organisms (X_{NOO}) producing nitrate. Ammonium oxidation is mostly performed by chemolithoautotrophic ammonium-oxidizing bacteria that use ammonium as their energy and nitrogen source and inorganic carbon as the carbon source. Ammonium oxidation has been reported to be performed also by certain Archea (X_{AOA}, Könneke *et al.,* 2005) and by heterotrophic bacteria (van Niel *et al.,* 1993), showing that the diversity of ammonium-oxidizing organisms is larger than previously assumed. However, due to the dominance of the chemolithoautotrophic pathway performed by X_{AOO} in wastewater treatment systems, this is the ammonium oxidation process considered in this chapter.

At the microbial process level, the oxidation of ammonium to nitrite proceeds through the formation of hydroxylamine (NH_2OH) as the intermediate via the enzyme ammonia monooxygenase (AMO):

$$NH_4^+ + O_2 + H^+ + 2e^- \rightarrow NH_2OH + H_2O \qquad \text{Eq. 2.4.1}$$

Hydroxylamine is then further oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO):

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \qquad \text{Eq. 2.4.2}$$

The combination of the two redox processes is known as nitritation:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \qquad \text{Eq. 2.4.3}$$

This equation represents the catabolic macro-chemical reaction equation of the nitritation process. When the anabolism is also considered, then the metabolic (catabolism plus anabolism) macro-chemical reaction equation becomes:

$$NH_4^+ + 1.383O_2 + 0.09HCO_3^- \rightarrow 0.982NO_2^- + 1.036H_2O + 0.018C_5H_7O_2N + 1.892H^+ \qquad \text{Eq. 2.4.4}$$

As presented in the equation above, about 2 moles of protons are produced per mole of ammonium oxidized. The carbonate system is usually the pH buffer available in the wastewater that neutralizes the production of protons through CO_2 stripping. When the carbonate buffer, usually measured in terms of alkalinity as calcium carbonate equivalents ($CaCO_3$, meq L^{-1}), is not available or is insufficient in the wastewater (e.g. in the case of municipal wastewater, alkalinity lower than about 100 mg $CaCO_3$ L^{-1} or 2 meq L^{-1}), then the pH may drop below pH 7.0 (Ekama and Wenzel, 2008).

In the second stage of the nitrification process, also known as the nitratation process, nitrite is oxidized to nitrate by X_{NOO} by means of the enzyme nitrite oxidoreductase (Nir):

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \qquad \text{Eq. 2.4.5}$$

X_{NOO} are aerobic chemolithoautotrophic bacteria using, for synthesis, inorganic carbon as a carbon source (e.g. HCO_3^-) and ammonium as a nitrogen source. When including bacterial growth in the above equation, the following metabolic macro chemical reaction equation is obtained:

$$NO_2^- + 0.003NH_4^+ + 0.485O_2 + 0.015HCO_3^- + 0.012H^+ \rightarrow NO_3^- + 0.009H_2O + 0.003C_5H_7O_2N \qquad \text{Eq. 2.4.6}$$

The combination of the nitritation process performed by X_{AOO} and the nitratation process performed by X_{NOO} constitutes the nitrification process. The nitrification stoichiometry can also be expressed according to the standard ASM (activated sludge model) as:

$$\left(\frac{1}{Y_{ANO}} + i_{N,ANO}\right)\cdot S_{NHx} + \frac{4.75 - Y_{ANO}}{Y_{ANO}}\cdot S_{O2} + \left(\frac{i_{N,ANO}}{14} + \frac{2}{14\cdot Y_{ANO}}\right)\cdot S_{IC} \rightarrow X_{ANO} + \frac{1}{Y_{ANO}}\cdot S_{NO3} \qquad \text{Eq. 2.4.7}$$

Where, S_{NO3} is the nitrate concentration (mg N L^{-1}); S_{IC} is the alkalinity concentration (mmol L^{-1}), S_{O2} is the DO concentration (mg O_2 L^{-1}), S_{NHx} is the ammonium concentration (mg N L^{-1}), X_{ANO} is the concentration of nitrifying organisms (mg COD L^{-1}), Y_{ANO} is the growth yield of nitrifying microorganisms (g COD g N^{-1}), and

$i_{N,ANO}$ is the nitrogen content in the nitrifying organism's cells (g N g COD^{-1}).

2.4.1.2 Denitrification

In the 1850s, first Reiset (1856) and then Pasteur (1859) reported that the reduction of nitrate was of a biological nature, marking the beginning of research into the biological nitrogen cycle. Even if Pasteur erroneously attributed nitrate reduction to 'lactic yeast', he understood the role of organics. Reiset (1856) observed that nitrogen is released into the atmosphere during the decay of plant and animal residues. Shortly after, Gayon and Dupetit (1883) named the process denitrification, organics were experimentally proven to be required in the process (Munro, 1886), and nitrite, nitric oxide (NO) and nitrous oxide (N_2O) were identified as intermediates (Payne, 1986). It was observed that not only bacteria but also eukaryotes and archaea can grow on the energy gained by the oxidation of organics or inorganic substrates coupled with the reduction of nitrate to nitrite, NO, N_2O and finally dinitrogen gas (N_2) (Risgaard-Petersen *et al*., 2006; Pina-Choa *et al.,* 2010). Certain microorganisms capable of performing heterotrophic nitrification were also shown to be able to carry out denitrification under aerobic and anoxic conditions, the so-called *aerobic denitrification* (Robertson *et al.,* 1995). Denitrification may also proceed without N_2O as an intermediate as in the recently discovered denitrification using methane as the electron donor (Ettwig *et al.,* 2010). Nitrifier denitrification where X_{AOO} reduce nitrite to N_2O has also been reported (Bock *et al.,* 1995). In contrast to other microorganisms in the nitrogen cycle (e.g. X_{AOO}, X_{NOO}, anammox), several ordinary heterotrophic organisms (X_{OHO}) are facultative denitrifiers that preferentially use oxygen as an electron acceptor due to the higher energy yield and only switch to denitrification when low oxygen levels prevail in the presence of nitrate or nitrite (Zumft, 1997). Independent of the electron donor used, the overall biochemical pathway for denitrification involves the same enzymes in each of these reduction steps from nitrate to dinitrogen gas: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS):

$$NO_3^-(aq) \xrightarrow{NAR} NO_2^-(aq) \xrightarrow{NIR} NO(g) \xrightarrow{NOR} N_2O(g) \xrightarrow{NOS} N_2(g) \quad \text{Eq. 2.4.8}$$

If either the denitrifying microorganisms do not express all the enzymes for the complete denitrification chain or alternatively under certain environmental conditions, then the intermediates NO and N_2O can be emitted, both of which have a negative impact on the environment due to their toxicity and direct or indirect contribution to the greenhouse effect.

In wastewater treatment systems, biological nitrogen removal is mostly carried out by X_{OHO} using organic matter as the electron donor. When the organics naturally present in the wastewater are not sufficient to achieve complete denitrification, usually external electron donors (such as acetic acid or methanol, among others) are dosed. Due to its prevalence in wastewater applications, only denitrification catalysed by X_{OHO} (heterotrophic denitrification) is considered in this chapter, which occurs by the following generic catabolic pathway (Mateju *et al.,* 1992):

$$NO_3^- + 1.25CH_2O \rightarrow 0.5N_2 + OH^- + 0.75H_2 + 1.25CO_2 \quad \text{Eq. 2.4.9}$$

This equation illustrates that heterotrophic denitrification renders the environment more alkaline due to the production of hydroxide ions.

X_{OHO} can use both nitrate and nitrite as an electron acceptor. The process of nitrate reduction to dinitrogen gas is called denitrification, while the process of nitrite reduction to dinitrogen gas is called denitritation. According to standard activated sludge model (ASM) notation, when a generic soluble and biodegradable substrate (S_B) is used as the carbon source, the denitrification and denitritation stoichiometry can be described as follows, respectively:

$$\frac{1}{Y_{OHO,Ax}} \cdot S_B + \frac{1 - Y_{OHO,Ax}}{2.86 \cdot Y_{OHO,Ax}} \cdot S_{NO3} + i_{N,OHO} \cdot S_{NHx} \rightarrow X_{OHO} + \left(\frac{1 - Y_{OHO,Ax}}{2.86 \cdot 14 \cdot Y_{OHO,Ax}} - \frac{i_{N,OHO}}{14} \right) \cdot S_{IC} + \frac{1 - Y_{OHO,Ax}}{2.86 \cdot Y_{OHO,Ax}} \cdot S_{N2} \quad \text{Eq. 2.4.10}$$

$$\frac{1}{Y_{OHO,Ax}} \cdot S_B + \frac{1 - Y_{OHO,Ax}}{1.71 \cdot Y_{OHO,Ax}} \cdot S_{NO2} + i_{N,OHO} \cdot S_{NHx} \rightarrow X_{OHO} + \left(\frac{1 - Y_{OHO,Ax}}{1.71 \cdot 14 \cdot Y_{OHO,Ax}} - \frac{i_{N,OHO}}{14} \right) \cdot S_{IC} + \frac{1 - Y_{OHO,Ax}}{1.71 \cdot Y_{OHO,Ax}} \cdot S_{N2} \quad \text{Eq. 2.4.11}$$

Where, $Y_{OHO,Ax}$ is the heterotrophic growth yield under anoxic conditions (g COD g COD^{-1}), X_{OHO} is the concentration of heterotrophic microorganisms (mg COD L^{-1}), S_{NHx} is the ammonium concentration (mg N L^{-1}), S_{NO2} the nitrite concentration (mg N L^{-1}), S_{NO3} the

nitrate concentration (mg N L^{-1}), S_{N2} is the concentration of dissolved dinitrogen gas (mg N L^{-1}), and S_{IC} is the alkalinity concentration (mmol L^{-1}).

2.4.1.3 Anaerobic ammonium oxidation (anammox)

The anammox process can be regarded as a peculiar type of denitrification, in which the oxidation of ammonium is coupled to the reduction of nitrite. Its discovery in the 1990s radically changed the understanding of the biological nitrogen cycle (Kuypers *et al.,* 2005), refuting the conventional assumption at that time that ammonium was chemically inert, and that its oxidation required oxygen and a mixed-function oxygenase enzyme (van de Graaf *et al.,* 1996). Anammox bacteria belong to the genus *Planctomycetes* and have been detected in several wastewater treatment plants and natural environments all around the world, showing their ubiquitous distribution. Ammonium, nitrite and bicarbonate are the main substrates in the anammox process (van de Graaf *et al.,* 1996). The catabolic reaction catalysed by anammox bacteria couples the nitrogen atoms from ammonium and nitrite to form dinitrogen gas (N_2):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \text{Eq. 2.4.12}$$

In the absence of oxygen, anammox bacteria activate the stable ammonium molecule through the oxidizing power of nitric oxide (NO). Briefly, anaerobic ammonium oxidation is a three-step process with NO and hydrazine as intermediates: first, nitrite is reduced to NO by the enzyme nitrite oxidoreductase (Nir), then the produced NO reacts with ammonium to form hydrazine (N_2H_4), catalysed by the unique hydrazine synthase enzyme (HZS), and finally hydrazine is oxidized to N_2 by hydrazine dehydrogenase (HDH) (Kartal *et al.,* 2011). Anammox bacteria are autotrophs and thus make use of inorganic carbon as the carbon source for the production of biomass. In the anabolic reaction the reducing equivalents for the reduction of inorganic carbon originate from the oxidation of nitrite to nitrate as illustrated in the equation below (ammonium is considered as the N source):

$$HCO_3^- + 2.1NO_2^- + 0.2NH_4^+ + 0.8H^+ \rightarrow CH_{1.8}O_{0.5}N_{0.2} + 2.1NO_3^- + 0.4H_2O \qquad \text{Eq. 2.4.13}$$

The metabolic macro chemical reaction equation of the anammox process is still subject to debate. Due to the difficulties in cultivating a pure culture of anammox bacteria, stoichiometric equations derived by substrates/products via mass balance are intrinsically not completely precise (Lotti *et al.,* 2014). However, the first reaction stoichiometry reported by Strous and co-authors (1998) is widely used for reactor design and operation purposes:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.066CH_2O_{0.5}N_{0.15} + 0.26NO_3^- + 2.03H_2O \qquad \text{Eq. 2.4.14}$$

Since nitrogen is usually present in wastewater as ammonium but the anammox metabolism requires both ammonium and nitrite as substrates, the anammox process has to be combined with another process to generate the required nitrite. For this purpose, the partial nitritation (PN) process is usually applied. Nitrogen removal processes based on the combination of PN and anammox process are currently part of the state of the art with about 100 full-scale implementations worldwide treating mostly the effluent from anaerobic sludge digesters as well as a variety of ammonium-rich municipal and industrial wastewaters: leather tanning, food processing, and the semiconductor, fermentation, yeast production, distilling, and winemaking industries (Lackner *et al.,* 2014). Furthermore, encouraging results were reported for pilot-scale installations treating black water digestate (de Graaff *et al.,* 2011), digested manure (Villegas *et al.,* 2011), urine (Udert *et al.,* 2008) and pharmaceutical wastewaters (Tang *et al.,* 2011). Recently, positive results have also been obtained in the treatment of aerobically pre-treated sewage, opening up new perspectives for converting municipal WWTPs from energy-depleting to energy-generating systems (Lotti *et al.,* 2015a).

2.4.2 Process-tracking alternatives

According to the stoichiometry of the processes involved in nitrogen removal, as presented in Section 2.4.1, various alternatives are available to assess the process kinetics and stoichiometric parameters of interest during a batch test, such as:

- Chemical tracking by assessing the nitrite, nitrate or ammonium concentrations over time; the choice of the optimal chemical species to be tracked depends on the specific process of interest.
- Titrimetric tracking by applying pH-static titration to those processes that relevantly affect the solution pH.
- Manometric tracking, applicable to processes involving soluble gaseous species of low solubility such as N_2.
- Respirometry, applicable to aerobic processes that affect the DO concentrations.

The last alternative is presented not in this chapter, but in Chapter 3 on Respirometry, which is fully dedicated to this technique.

The other tracking alternatives are briefly presented hereafter.

2.4.2.1 Chemical tracking

Tracking the concentration over time of substrates and products is the most common way to assess the kinetics of a process.

When nitrification is to be tracked, ammonium and nitrate concentrations are monitored over time. For the separate assessment of the nitritation or nitratation rates, ammonium and nitrite or nitrite and nitrate concentrations have to be measured over time. Finally, when nitritation and nitratation rates have to be assessed simultaneously, ammonium, nitrite and nitrate concentrations need to be monitored over time.

For denitrification, the most common batch test that applies the chemical tracking procedure is the so-called nitrate uptake rate (NUR) test. NUR tests make it possible to assess several parameters of practical interest, such as nitrate utilization rates, the utilization of organics for denitrification and the anoxic biomass yield coefficient (Naidoo *et al.,* 1998; Kujawa and Klapwijk, 1999). As described in Section 2.2.4, samples are taken during the course of the batch tests and then chemical analyses of the main substrates (e.g. nitrite, nitrate and COD) are performed in order to get sufficient information for the assessment of relevant kinetic and stoichiometric parameters.

Finally, when this method is applied to the evaluation of the anammox process kinetics, ammonium, nitrite and nitrate concentrations are monitored over time. Besides the anammox process rate, this method also makes it possible to assess stoichiometric parameters of interest such as the ratio between nitrite and ammonium consumption (NO_2/NH_4 ratio) and the ratio between nitrate production and ammonium consumption (NO_3/NH_4 ratio) which can be calculated as the ratio of the corresponding conversion rates (Lotti *et al.,* 2014).

2.4.2.2 Titrimetric tracking

The pH-static titration technique consists of the controlled addition of an appropriately diluted solution of acid or base to maintain a constant pH (therefore 'static' pH) in a biological system where the pH is affected by different reactions. Under these conditions, the titration rate is proportional to the reaction rate via a stoichiometric factor. In principle, this technique is applicable to any biological or physical-chemical reaction affecting the proton concentration (thus, linked with pH), i.e. any reaction converting neutral substrates into acid or basic products, or acid or basic substrates into neutral products. There are several biological reactions of environmental interest that affect the pH of the suspension where they take place. Attempts to use pH-static titration have been mainly focused on nitrification (Gernaey *et al.,* 1997, 1998; Massone *et al.,* 1998) and denitrification (Massone *et al.,* 1996; Rozzi *et al.,* 1997; Bogaert *et al.,* 1997; Foxon *et al.,* 2002). Although these are consolidated applications of the pH-static titration technique, others can be foreseen, such as those involving the $CO_2/HCO_3^-/CO_3^{2-}$ equilibria, e.g. the heterotrophic degradation of organic substrates that produces CO_2 (Ficara and Rozzi, 2004) and acetoclastic methanogenesis that produces bicarbonate (Rozzi *et al.,* 2002).

Nitrification monitoring is the first and most consolidated application of this technique since the relationship between the ammonium oxidised and the proton produced Y_{NH4_H+} can be calculated from the reaction stoichiometry, as assumed by ASM1 (Henze *et al.,* 2000):

$$Y_{NH\_H+} = \frac{14}{2 + i_{N,ANO} \cdot Y_{ANO}} \approx 6.92 \text{ g N mol (protons)}^{-1} \quad \text{Eq. 2.4.15}$$

The N/H ratio is therefore the stoichiometric factor that allows the conversion of the titration rate into the ammonium consumption rate. The pH-static titration technique can also be applied to denitrification since this process is a 'pH-affecting reaction'. However, the assessment of the ratio between nitrite or nitrate consumption and proton production Y_{NOx_H+} based on stoichiometry is not as straightforward as for nitrification, mostly because it depends on many more factors, such as the carbon source, the sludge characteristics, and the pH set point. To theoretically calculate (Y_{NOx_H+}, a conceptual model was proposed by Petersen *et al.* (2002), which considers that the following four processes have a pH-dependent effect on proton production during denitrification: (*i*) uptake of weak organic acids as carbon source, (*ii*) uptake of nitrate, (*iii*) uptake of ammonia for cell synthesis, and (*iv*) production of carbon dioxide from organic carbon oxidation. Based

on these assumptions, the following reaction stoichiometry was proposed to assess the ratio between the net proton production and nitrate consumption:

$$\frac{1}{Y_{OHO,Ax}} \cdot S_B + \frac{1 - Y_{OHO,Ax}}{\beta \cdot Y_{OHO,Ax}} \cdot S_{NOx} + i_{N,OHO} \cdot S_{NHx} \rightarrow X_{OHO} + \frac{1 - Y_{OHO,Ax}}{\beta \cdot Y_{OHO,Ax}} \cdot S_{N2} + \left[-\frac{a}{C \cdot Y_{OHO,Ax}} - \frac{1 - Y_{OHO,Ax}}{\beta \cdot 14 \cdot Y_{OHO,Ax}} + \frac{b \cdot i_{N,OHO}}{14} + \frac{C \cdot (1 - Y_{OHO,Ax}) \cdot x}{C \cdot Y_{OHO,Ax}} \right] \cdot H^+$$

Eq. 2.4.16

Where, S_{NOX} is the nitrite or nitrate concentration; x is the number of carbon moles per mole of organic substrate, C is a factor (in g COD mol^{-1} organic substrate) to express the organic carbon in COD units, β is the oxygen equivalent of oxidized nitrogen; and a, b, c are pH-dependent factors which take into account the dissociation equilibria of weak acids/bases (a for organic acids - HA, b for carbonic acid, and c for ammonium):

$$a = \frac{[A^-]}{[HA] + [A^-]} = \frac{10^{-pKa}}{10^{-pH} + 10^{-pKa}}$$ Eq. 2.4.17

$$b = \frac{10^{pH - pK1} \cdot (1 + 2 \cdot 10^{pH - pK2})}{1 + 10^{pH - pK1} \cdot (1 + 10^{pH - pK2})}$$ Eq. 2.4.18

$$c = \frac{[NH_4^+]}{[NH_4^+] + [NH_3]} = \frac{10^{-pH}}{10^{-pH} + 10^{-pK_{NH4}}}$$ Eq. 2.4.19

Where, pK_a is the dissociation constant for acetic acid (4.75 at 25 °C), pK_1 the dissociation constant for carbonic acid (6.352 at 25 °C), pK_2 the dissociation constant for bicarbonate (10.33 at 25 °C), and pK_{NH4} the dissociation constant for ammonium (9.25 at 25 °C).

By substituting the correct value of β (i.e. 2.86 g COD N^{-1} for $N\text{-}NO_3$ and 1.72 g COD N^{-1} for $N\text{-}NO_2$), it follows that Y_{NOx_H+}, in g N mol^{-1}, in the presence of nitrate, Y_{NO3_H+}, or nitrite, Y_{NO2_H+}, as an electron acceptor, can be expressed as:

$$Y_{NO3\_H+} = \frac{1 - Y_{OHO,Ax}}{2.86 \cdot Y_{OHO,Ax}} \cdot \left[-\frac{a}{C \cdot Y_{OHO,Ax}} - \frac{1 - Y_{OHO,Ax}}{2.86 \cdot 14 \cdot Y_{OHO,Ax}} + \frac{c \cdot i_{N,OHO}}{14} + \frac{b \cdot (1 - Y_{OHO,Ax}) \cdot x}{C \cdot Y_{OHO,Ax}} \right]^{-1}$$ Eq. 2.4.20

$$Y_{NO2\_H+} = \frac{1 - Y_{OHO,Ax}}{1.72 \cdot Y_{OHO,Ax}} \cdot \left[-\frac{a}{C \cdot Y_{OHO,Ax}} - \frac{1 - Y_{OHO,Ax}}{1.72 \cdot 14 \cdot Y_{OHO,Ax}} + \frac{c \cdot i_{N,OHO}}{14} + \frac{b \cdot (1 - Y_{OHO,Ax}) \cdot x}{C \cdot Y_{OHO,Ax}} \right]^{-1}$$ Eq. 2.4.21

These equations show that the assessment of Y_{NOx_H+} makes it necessary to know the chemical composition of the carbon source (C and x), which is seldom the case in practical applications, and the anoxic biomass growth yield coefficient, $Y_{OHO,Ax}$. As such, its evaluation is theoretically possible, but difficult in practice.

Fortunately, Y_{NOx_H+} can also be experimentally evaluated by measuring the amount of titration solution (normally acid) dosed under pH-static conditions to denitrify a known amount of nitrite or nitrate and in the presence of the carbon source of interest. Once the Y_{NOx_H+} ratio is measured, the titration rate can be easily converted into the nitrate or nitrite uptake rate.

Theoretically, even the anammox process can be monitored by pH-static titration. However, there is very little available experience of this, and therefore this alternative is not discussed in this chapter.

2.4.2.3 Manometric tracking

According to this technique, the rate of a bioprocess that produces a poorly soluble gaseous component is proportional to the rate of increase in pressure, provided that the bio-reaction takes place in a gas-tight reactor. The relationship between the generated overpressure, P(t), and the volumetric gas production, $V_G(t)$, can be obtained by assuming that the gas transfer from the liquid to the gas phase is not rate-limiting (sludge mixing allows the quick transfer of gaseous species) and that no relevant amounts of the gaseous species remain in solution. Under these conditions and at constant temperature, according to the gas law, the following relationship applies:

$$V_G(t) = \frac{P(t) - P_{atm}}{P_{atm}} \cdot V_{HS}$$ Eq. 2.4.22

Where, V_{HS} is the volume of the headspace in the reactor and P_{atm} is the atmospheric pressure.

This measuring principle was proven to be applicable and advantageous in the monitoring of denitrification (Sánchez *et al.,* 2000; Ficara *et al.,* 2009) and of anaerobic ammonia oxidation (Dapena Mora *et al.,* 2007;

Scaglione *et al.,* 2009; Bettazzi *et al.,* 2010; Lotti *et al.,* 2012) since both processes produce dinitrogen gas. As for denitrification, a CO_2 adsorbent should be used that is typically located in the gas headspace (e.g. NaOH pellets) so that overpressure data are only related to the release of N_2.

2.4.3 Experimental setup

2.4.3.1 Reactors

Independently of the technology applied to remove nitrogen from wastewater, to assess the performance of the biological nitrogen removal process, batch activity tests can be carried out under aerobic (nitrification) or anoxic conditions (denitrification and anammox) depending upon the parameters of interest and nature of the study. In any case, the reactor(s) used for the execution of tests must have the required means to: (*i*) avoid oxygen intrusion under anoxic conditions, (*ii*) secure satisfactory S_{O2} availability under aerobic conditions (e.g. S_{O2} higher than 2 mg L^{-1}), (*iii*) provide satisfactory mixing conditions, (*iv*) maintain an adequate and desirable temperature, (*v*) provide precise pH control, and (*vi*) have additional ports for sample collection and addition of influent, solutions, gases and any other liquid media or substrate used in the test. For the requirements needed to ensure proper anoxic conditions, aerobic conditions, mixing, temperature control, pH control and sampling and dosing ports during the execution of the tests, the reader is referred to Section 2.2.2.1. However, when titrimetric or manometric experiments are to be performed, special apparatus should be available, as described below.

2.4.3.2 Instrumentation for titrimetric tests

To perform set point titration tests, an automated titration unit is required. As for respirometers, such systems are readily available on the market. However, they can be easily implemented by using conventional laboratory equipment and basic signal acquisition and control units. Specifically, an automated titration unit should be made up of the following components (see Figure 2.4.1):

- A reaction vessel: a well-mixed reactor with a thermostat or temperature control to host the activated sludge sample with an operative volume of 0.5 to 1.5 L. The reaction vessel does not need to be gas-tight when performing nitrification tests. However, a reduced solid-liquid contact area is preferable to limit gas-liquid transfer of oxygen and carbon dioxide. Gas tightness for proper anoxic operation is required when performing denitrification tests.
- Probes to assess: temperature (with a resolution of 0.1 °C), pH (with a resolution of 0.01 pH units) and, possibly, DO (± 0.02 mg L^{-1} around the selected set point value).
- An aeration system (for aerobic processes), typically with an aeration capacity of 50-200 L L^{-1} h^{-1}, and stone diffuser for fine bubble aeration;
- An automated titration system capable of maintaining the pH within a narrow range (e.g. at a defined pH set point ± 0.02) and to record the volume of the titration solution dosed over time with a suggested resolution of 0.1 mL and a minimum logging frequency of 1 datum per minute. Manual recording of the added titration solution volume can also be obtained by storing the titration solution in a graduated cylinder and by manually reading the remaining volume at regular intervals (every few minutes for nitrification tests) or by installing the solution on a balance with its weight either read or recorded automatically. A solution of 0.05-0.02 N NaOH can be used as an alkaline titration solution, while a 0.05-0.02 N HCl solution can serve as an acidic titration solution.

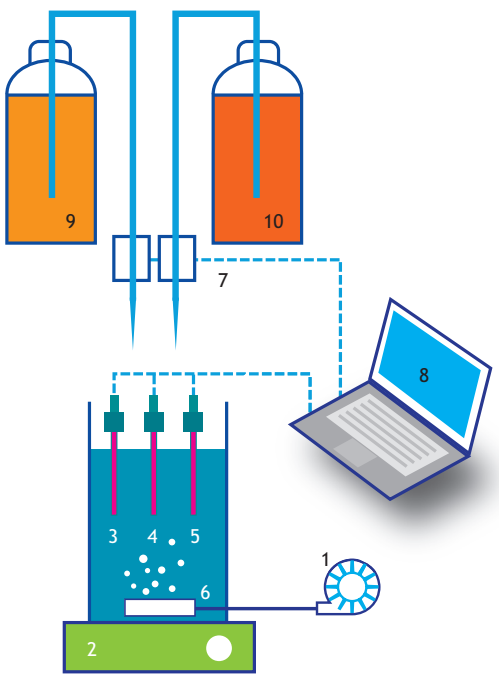

Figure 2.4.1 Scheme of a pH-static titration system: 1. aerator; 2. mixer; 3. temperature probe; 4. pH probe; 5. DO probe; 6. reaction vessel; 7. titration-solution dosing system; 8. signal acquisition and recording; 9. alkaline titration solution; 10. acidic titration solution.

When aerobic bioprocesses are involved, the pH-static system can be conveniently upgraded into a pH/DO-stat system, in which a secondary titration unit provides an H_2O_2 diluted solution, serving as an oxygenated titration solution. For this purpose, the system described in Figure 2.4.1 should be upgraded by integrating:

- A DO probe, with a minimum resolution of 0.1 mg L^{-1}.
- An additional automated dosing or titration system capable of maintaining the S_{O2} within a narrow range (± 0.1 mg L^{-1} around the selected set point value). A 0.05-0.2 M H_2O_2 solution is appropriate when performing pH/DO-static titration tests on conventional activated sludge samples. When dosed, the H_2O_2 solution will be converted into molecular oxygen (O_2) and water by peroxydases produced by aerobic bacteria to counteract oxidative stress, and thus making oxygen available for bacterial respiration. As a matter of fact, it has been observed that diluted H_2O_2 solutions can be used for short-term respirometric tests without significant bacteria inhibition (e.g. Ficara *et al.,* 2000).

The scope of this DO-static titration unit is to maintain the DO value at a predefined set point level (DO-set point) by titrating the oxygenated titration solution, thus meeting the following objectives:

- To maintain the desired redox condition without the need for air bubbling; this makes it possible to avoid CO_2 stripping which is a pH-affecting process that overlaps with other targeted pH-affecting reactions.
- To assess the oxygen consumption rate of the reaction that, under DO-static operation, equals the titration rate of the oxygenated titration solution. This is additional information that can be used to check or complement the titration rate of the alkaline/acidic solution, as described in detail later on in this chapter.

2.4.3.3 Instrumentation for manometric tests

Tests should be performed by using gas-tight apparatus. Typically, systems applied to perform BOD tests are used. The minimum requirements are the following:

- A glass bottle with (see Figure 2.4.2):
  a. A working volume of around 1 L.
  b. Two lateral openings, sealed by rubber septa kept in place by plastic or aluminium gear, for substrate injections and gas flushing/discharge.
  c. A container for NaOH pellets located in the bottle headspace and serving as a CO_2 trap.
  d. A manometric measuring device, possibly featured with a data logger, and fixed on the top of a glass bottle with a resolution of 1-3 mbar.
- A constant temperature incubator that limits temperature oscillations to ± 0.2 °C. It is mandatory that temperature is very well controlled during the course of the test since temperature variations cause changes in the overpressure values that are not associated with gas release and this would therefore result in data noise.
- A magnetic stirrer that can operate at around 100-200 rpm. Alternatively, a thermostatic orbital shaker can be used to ensure both temperature control and mixing. In the case of anammox, a magnetic stirrer is advisable only in the case of suspended anammox biomass (100-200 rpm), while for both hybrid, biofilm on carriers and granular anammox biomass types (Hu *et al.,* 2013), a shaker is preferred in order to avoid deterioration of the anammox biofilm due to the shear forces caused by the magnetic stirrer.

Figure 2.4.2 Commercially available apparatus to perform manometric tests for denitrification purposes (photo: Lotti, 2016).

2.4.3.4 Activated sludge sample collection

The sampling time and location of an activated sludge sample performing nitrification and denitrification is highly dependent on the type of batch activity test to be conducted. The removal of nitrogen operated via nitrification and denitrification processes is based on the alternating aerobic-anoxic conditions. Thus, preferably, a fresh sample should be collected at the end of the relative reaction stage: aerobic for nitrification, anoxic for denitrification. Certainly, the sampling location will depend on the system configuration. For instance, the sampling time and location of an activated sludge sample from a PN/anammox system depends on the type of technology used; obviously when PN and anammox processes are divided into two separate stages, anammox biomass should be sampled by the anammox stage. In full- and pilot-scale wastewater treatment plants, the physical boundaries between stages must be identified prior to sampling. In extreme cases, where the phases are not (physically) well defined, the redox limits or boundaries need to be determined with the use of a DO meter, redox meter and/or by determination of the nitrate and nitrite concentrations. In lab-scale systems (usually operated on a time-base mode), the sample collection can be relatively easier, since the reaction time defines the length of the stages. To obtain homogenous and representative samples, the sludge samples must be collected in sampling spots where well-mixed conditions take place.

When anammox is the targeted process, then sampling from the outlet of the anammox tank is typically adequate. This sampling point ensures that the sludge samples collected contain limited amounts of residual ammonium/nitrite concentrations. When granular anammox biomass is considered, the outlet of the anammox tank normally contains very few granules because a granular system is usually equipped with a biomass retention system (e.g. a three-phase separator, hydro-cyclone, settling phase in a sequencing batch reactor (SBR) cycle, etc.). In the case of anammox granular systems then sampling the mixed liquor directly from the anammox tank may be adequate in continuously operated systems (CSTR, continuous stirred tank reactor), while for SBR systems, sampling should be performed before the settling phase when the reactor is completely mixed to ensure completely mixed conditions and the presence of limited amounts of residual ammonium/nitrite concentrations.

Ideally, batch activity tests must be performed as soon as possible after sample collection. In lab-scale systems, in principle, this should not be a problem if the batch activity tests are performed in the same laboratory and their execution is coordinated and synchronized with the operations of the lab reactor. Also, at full- and pilot-scale treatment plants, batch activity tests can be performed *in situ* shortly after mixed liquor collection if the sewage plant laboratory is conditioned and equipped with the required experimental and analytical equipment. If the batch activity tests cannot be performed *in situ* on the same day, a mixed liquor sample can be collected and transferred to the location where the tests will be executed. Thereafter, the sampling bucket can be properly stored and transported in a fridge or in ice box (below or close to 4 °C) under non-aerated conditions and the activity tests should be performed no later than 24 h after sampling. In order to avoid the creation of anaerobic conditions during storage and the undesired production of toxic sulphide through the reduction of sulphate, nitrate should be added to the mixed liquor at a final concentration of about 50-200 mg N L^{-1}. However, the availability of nitrate would promote endogenous biomass respiration. Therefore, it is important to stress that it is highly recommended to conduct the test as soon as possible after sample collection.

Especially after storage, the biomass present in the mixed liquor sample needs to be 'washed' to remove any added nitrate, 're-activated' and acclimatized to the target pH and temperature of interest prior to the execution of the batch activity tests. The washing step must be performed by using an appropriate 'washing medium' with a mineral composition that depends on the target microbial population, as indicated below. Tap water can also be used as a washing medium as long as its conductivity is similar to that of the cultivation medium. In any case, the *in situ* execution of the batch activity tests is preferable since this avoids the exposure of biomass to varying conditions. The total volume of activated sludge (mixed liquor) to be collected depends on the number of tests, reactor volume and total volume of samples to be collected to assess the biomass activity. Often, 10-20 L of activated sludge or mixed liquor from full-scale wastewater treatment plants is considered sufficient. On the other hand, samples collected from lab-scale reactors rarely reach more than 1 L because lab-scale systems are usually smaller (from 0.5 to 2.2 L and in certain cases up to 8-10 L) and the maximum volume that can be withdrawn from lab-scale reactors is often set by the daily withdrawal of the excess of sludge from the system. Since the maximum volume allowed to be withdrawn is

directly related to the applied solids retention time (SRT), which is defined by the growth rate of the organisms, particular attention must be paid when dealing with slow-growing organisms such as nitrifiers and anammox bacteria.

Suggestions on sampling scheduling and ideal storage times have been previously described (see Section 2.2.3) and should be carefully considered.

2.4.3.5 Activated sludge sample preparation

Generally speaking, activated sludge samples can be used as such or after specific adjustments in pH (with or without the presence of a pH buffer), temperature, ammonium/nitrate/nitrite concentration, carbon source concentration, and X_{VSS}. For conventional activated sludge samples from wastewater treatment plants treating urban wastewater, a sample which has a X_{VSS} around 2-4 g VSS L^{-1} would be ideal. For anammox sludge, X_{VSS} around 5-10 g VSS L^{-1} will be preferable. For very diluted or concentrated sludge samples, a pre-concentration step (e.g. by decanting into an Imhoff cone for 30 min or by centrifuging at 4,000 rpm for a few minutes) or dilution with the secondary effluent of the same wastewater treatment plant may be helpful. This will avoid the occurrence of too slow or too fast conversion rates.

When such procedures are implemented on anoxic or anaerobic activated sludge samples, N_2 sparging should be performed immediately afterwards in order to re-establish proper anoxic/anaerobic conditions. When the anammox process is considered, a mixture of N_2/CO_2 gases (usually 95/5 % is used in practice) can be used for sparging instead of N_2 in order to avoid excessive CO_2 stripping which would cause a pH increase and may limit anammox activity during the batch test due to limiting inorganic carbon concentration. As for pH and temperature, in principle, the closer the set point pH value is to the typical operational pH of the plant, the more the resulting process rate will be representative of the operational process rate. The same concept applies to the selection of the temperature value. Since the effect of the anammox process on the pH is rather limited (0.13 mole of protons consumed per mole of ammonium converted), limited variations on the pH are expected during the execution of a batch test (e.g. from 7.5 to 7.9 according to Lotti *et al*., 2012). Nevertheless a pH buffer such as Hepes (N-2-hydroxyethyl-piperazine-N0-2-ethane sulfonic acid) or phosphate can be used to maintain a constant pH throughout the duration of the batch test (Dapena-Mora *et al.,* 2007; Lotti *et al.,* 2012). While a Hepes buffer can be used at concentrations up to 25 mM without affecting anammox activity (Lotti *et al.,* 2012), the concentration of the phosphate buffer should be carefully decided since it may result in process inhibition (Dapena-Mora *et al.,* 2007; Oshiki *et al.,* 2011). Previous reports have shown that a phosphate buffer concentration of 5.3 mM is suitable for the conduction of anammox batch tests (Dapena-Mora *et al.,* 2007; Lotti *et al.,* 2012).

In general, the objective of the sampling and experimental campaigns should be to minimize as much as possible the need for transportation, cooling, storage and reactivation of the sludge. Whenever possible, it is advisable to use 'fresh' sludge (and substrate/media). When actual operational conditions are to be tested, then the corresponding batch activity tests must be executed right away after sludge collection with the minimum adjustments of the operational conditions (e.g. for pH and temperature). When the batch tests cannot be performed *in situ* or shortly after collection, sludge samples must be stored at around 4 °C for preservation purposes during transportation and storage. In the case of anammox biomass, ambient temperature can be adopted for preservation purposes during transportation and storage. Storage at ambient temperature is suggested to avoid temperature shocks from the usual operative temperature of an anammox system (25-35 °C) to 4 °C, which is usually considered an adequate storage temperature for conventional activated sludge samples. Under these circumstances, the batch activity tests should preferably be executed in less than 24 h after sludge collection and after 'reactivation' by keeping the sludge at the pH and temperature of interest (after N_2 flushing in case of denitrification and anammox).

The addition of limited amounts of the substrate can favour bacterial metabolic reactivation. However, the preparation of the activated sludge is test-dependent and specific suggestions/recommendations are described in the following paragraphs.

2.4.3.6 Substrate

When real wastewater (either raw or settled) is used for the execution of activity tests, it can be fed in a relatively straightforward manner to the reactor/fermenter. A rough filtration step (using 10 µm pore filter size) can be used to remove the remaining debris and large particles present in the raw wastewater.

If different carbon sources and concentrations are to be studied, the plant effluent can also be used to prepare

a semi-synthetic media containing a S_B concentration of between 50 and 100 mg COD L^{-1}.

For the execution of conventional denitrification tests, nitrate and nitrite solutions can be prepared to create the required anoxic conditions. For this purpose, different stock solutions can be prepared using nitrate- and nitrite-salts.

For the execution of anammox tests, ammonium and nitrite need to be dosed to the wastewater at the beginning of the test in order to provide the desired amount of substrate. For this purpose, different stock solutions can be prepared using ammonium- and nitrite-salts (e.g. 1-10 g N L^{-1}); the most commonly used are ammonium sulphate and sodium nitrite, respectively. Particular attention needs to be paid when considering the initial substrate concentrations. Nitrite in fact, besides being a substrate for the anammox process, is also an inhibitor (Lotti *et al.,* 2012; Puyol *et al.,* 2014). Initial nitrite concentration around 50-70 mg N L^{-1} is usually considered adequate. Nevertheless, lower initial nitrite concentrations (e.g. 10 mg N L^{-1}) are recommended if the anammox biomass originates from systems operated at very low (few mg N L^{-1}) nitrite concentrations (e.g. DEMON systems, Wett *et al.,* 2007). In fact, the nitrite inhibition effect and resilience seems to depend on the 'cultivation history' of the anammox biomass, being the cultures cultivated under strict nitrite limitation more prone to nitrite inhibition (Lotti *et al.,* 2012).

When tests are executed to assess the potential inhibitory or toxic effect of a given compound at different concentrations, concentrated stock solutions can be prepared and added during the test to obtain the concentrations of interest. Tests performed to assess whether the inhibitory or toxic effects are reversible must be carried out after washing the biomass to remove the inhibiting or toxic compound(s). Often, the washing step is performed by consecutive settling and re-suspension of the sludge sample in carbon-free and nitrite-free media (either fully synthetic or using a treated effluent after filtration) under anoxic conditions.

2.4.3.7 Analytical procedures

Analytical procedures of interest (NH_4, NO_2, NO_3, MLSS, MLVSS, COD, BOD) should be performed following standardized and commonly applied analytical protocols detailed in Standard Methods (APHA *et al.,* 2012).

If a specific carbon source is used, its determination should be performed according to the relevant analytical method. However, most of the time, the determination of soluble COD can be appropriate to follow the carbon source utilization.

2.4.3.8 Parameters of interest

Nitrification

The most significant kinetic parameter of the aerobic ammonium oxidation process (nitritation) performed by AOO is the maximum biomass-specific ammonium oxidation rate ($q_{AOO,NH4}$). Similarly, for the aerobic nitrite oxidation process (nitratation) performed by NOO, the main parameter of interest is the maximum biomass-specific nitrite oxidation rate ($q_{NOO,NO2\_NO3}$). Table 2.4.1 presents typical kinetic parameter values found in literature for both the aerobic ammonium and nitrite oxidation processes. Literature values of the biomass growth yield of both AOO and NOO are also reported in Table 2.4.1.

Table 2.4.1 Expected stoichiometric and kinetic parameters of interest for activated sludge performing aerobic ammonium and nitrite oxidation to nitrite and nitrate. The kinetic parameters are reported considering a reference temperature of 20 °C.

Aerobic ammonium oxidation–nitritation process		
$q_{AOO,NH4}$ g N g VSS^{-1} d^{-1}	Y_{AOO} g VSS g N^{-1}	Reference
0.11	0.14	Blackburne *et al.* (2007)
0.09	0.11	Jones *et al.* (2007)
0.24	0.13	Jubany *et al.* (2008)
0.27	0.15	Koch *et al.* (2000)
0.21	0.11	Lochtman (1995)
0.22	0.15	Wiesmann (1994)
Aerobic nitrite oxidation–nitratation process		
$q_{NOO,NO2\_NO3}$ g N g VSS^{-1} d^{-1}	Y_{NOO} g VSS g N^{-1}	Reference
0.21	0.07	Blackburne *et al.* (2007)
0.13	0.07	Jones *et al.* (2007)
0.39	0.06	Jubany *et al.* (2008)
1.78	0.02	Koch *et al.* (2000)
0.45	0.03	Lochtman (1995)
0.78	0.04	Wiesmann (1994)
1.07	0.03	Wik and Breitholtz (1996)

The kinetics reported in Table 2.4.1 refer to an operational temperature of 20 °C and were calculated from the original values according to the Arrhenius equation reported below.

$$k_S(T) = k_S(T_{ref}) \cdot \exp\left(\frac{E_{a,S} \cdot (T_K - T_{ref})}{R \cdot T_K \cdot T_{ref}}\right) \qquad \text{Eq. 2.4.23}$$

Where, $k_S(T)$ is the maximum biomass-specific consumption rate of the substrate S evaluated at the desired operative absolute temperature T_K (K), T_{ref} is the reference absolute temperature, R is the ideal gas constant (8.31 J mol^{-1} K^{-1}), $E_{a,S}$ is the activation energy of the considered bioprocess consuming the substrate S. $E_{a,NH4}$ = 68 kJ mol-NH_4^{-1} K^{-1} and $E_{a,NO2}$ = 44 kJ mol-NO_2^{-1} K^{-1} are typical activation energy values for the nitritation process performed by AOO and the nitratation process performed by NOO, respectively.

As it can be observed in Table 2.4.1, the aerobic consumption rates for ammonium and nitrite oxidation reported in literature can vary widely, especially for the nitratation process catalysed by NOO. The main reason can be the active biomass fraction present in the activated sludge biomass, conventionally referred to as the total MLVSS concentration. The larger the fraction of active nitrifying biomass, the higher the specific biomass conversion rate expected. In a conventional activated sludge system this can be directly related to the COD/N ratio in the influent, with the lower COD/N ratio corresponding to the higher fraction of active nitrifying biomass. As depicted in Table 2.4.1, the reported growth yield values are higher for the ammonium-oxidizing bacteria than for the nitrite-oxidizing bacteria.

Denitrification

To quantify the activity of OHOs under anoxic conditions, the relevant stoichiometric parameters and kinetic constants have to be known. As for stoichiometry, the most relevant parameter to be defined is the heterotrophic growth yield under anoxic conditions $Y_{OHO,Ax}$ (in g COD-biomass per g COD-substrate). Like the aerobic growth yield, this parameter may depend on various factors, such as the organic carbon source quantity and quality, and the environmental conditions. Typical values for this parameter are listed in Table 2.4.2. This parameter can be easily determined by setting up appropriately designed batch activity tests, as explained later on. When performing denitrification, the carbon source required per nitrate/nitrite to be removed (expressed as the COD/N ratio or as the C/N ratio) can be used instead of $Y_{OHO,Ax}$, given that a stoichiometric relationship exists between the two of them. The COD/N ratio represents the denitrification capacity of a carbon source or of a wastewater and can be a more practical parameter than the $Y_{OHO,Ax}$.

Table 2.4.2 Expected stoichiometric and kinetic parameters of interest for activated sludge wastewater treatment systems performing denitrification.

Parameter (symbol)	Remark	Value	Reference
Heterotrophic anoxic growth yield ($Y_{OHO,Ax}$)	Acetate	0.66 g COD g COD^{-1}	Ficara and Canziani (2007)
	Wastewater	0.50 g COD g COD^{-1}	Orhon *et al.* (1996)
	Acetate	0.66 g VSS g COD^{-1}	Kujawa and Klapwijk (1999)
	Ethanol	0.22 g VSS g COD^{-1}	Hallin *et al.* (1996)
	Methanol	0.18 g VSS g COD^{-1}	Tchobanoglous *et al.* (2003)
COD to nitrogen ratio (COD/N)	Methanol	4.6 g COD g N^{-1}	Bilanovic *et al.* (1999)
	Methanol	4.7 g COD g N^{-1}	Mokhayeri *et al.* (2006)
	Ethanol	3.5 g COD g N^{-1}	Mokhayeri *et al.* (2006)
	Acetate	3.4 g COD g N^{-1}	Mokhayeri *et al.* (2006)
Maximum biomass-specific denitrification rate (q_{NOx_N2})	Acetate,	10-19 mg N g VSS^{-1} h^{-1}	Ficara and Canziani (2007)
	Acetate, nitrite	15-28 mg N g VSS^{-1} h^{-1}	Ficara and Canziani (2007)
	Methanol, 13 °C	9.2 mg N g VSS^{-1} h^{-1}	Mokhayeri *et al.* (2008)
	Ethanol, 13 °C	30.4 mg N g VSS^{-1} h^{-1}	Mokhayeri *et al.* (2008)
	Acetate, 13 °C	31.7 mg N g VSS^{-1} h^{-1}	Mokhayeri *et al.* (2008)
	Acetate	1-3 mg N g VSS^{-1} h^{-1}	Kujawa and Klapwijk (1999)
	Acetate	2-10 mg N g VSS^{-1} h^{-1}	Henze (1991)

Regarding the growth kinetics, the specific substrate consumption (either nitrate/nitrite or COD) can be easily determined by batch activity tests. This value depends on the operational conditions used during the test (especially on temperature and the substrate's nature and concentrations). Therefore, these values should always be specified when reporting the results of a test. As reference conditions, the denitrification tests should be performed at 20 °C under non-limiting concentrations of carbon and nitrate/nitrite. This will allow the determination of the maximum specific denitrification rate. In practical applications, the maximum biomass specific denitrification rate q_{NOx_N2} is linked to the mixed liquor suspended solids (X_{TSS}) or, more commonly, to their volatile suspended solids content (X_{VSS}). Values reported in literature can be found in Table 2.4.2.

Anammox

The most significant kinetic and stoichiometric parameters of the anaerobic nitrogen removal process performed by anammox bacteria are the maximum specific biomass ammonium oxidation rate ($q_{AMX,\ NH4\_N2}$), the nitrite to ammonium ($Y_{NH4\_NO2,AMX}$) consumption ratio and the ratio between nitrate production and ammonium consumption ($Y_{NH4\_NO3,AMX}$). In Table 2.4.3 typical kinetic and stoichiometric parameters values found in literature are reported, together with the biomass growth yield.

Table 2.4.3 Stoichiometric and kinetic parameters of interest for anammox biomass performing the anaerobic ammonium oxidation process. The kinetic parameters are obtained at 30 °C. Biomass type: suspended (S), flocculent (F), granular (G). Reactor of origin: lab- (Lab) or full-scale (Full) reactor performing the anoxic stage of a 2-stage PN/anammox system (2-stage) or the 1-stage PN/anammox system (1-stage).

$q_{AMX,\ NH4\_N2}$ g N_2-N g VSS^{-1} d^{-1}	$Y_{AMX,NH4}$ C-mol NH_4-mol^{-1}	$Y_{NH4\_NO2,AMX}$ mol mol^{-1}	$Y_{NH4\_NO3,AMX}$ mol mol^{-1}	Biomass type	Reactor of origin	Reference
0.66	0.066	1.32	0.26	F	Lab, 2-stage	Strous *et al.* (1998)
0.22		1.27	0.34	G	Lab, 2-stage	Puyol *et al.* (2013)
0.22	0.105	1.28	0.37	F	Lab, 2-stage	Puyol *et al.* (2013)
2.01	0.071	1.22	0.21	S	Lab, 2-stage	Lotti *et al.* (2014)
3.38	0.071			S	Lab, 2-stage	Lotti *et al.* (2015b)
0.16				G	Full, 1-stage	Lotti *et al.* (2015c)
0.55				G	Full, 2-stage	Lotti *et al.* (2015c)

This parameter, even though it cannot be directly measured through batch tests, is useful to convert the specific biomass activity to growth rate values. Different from other bioprocesses described in this section, the anammox kinetics reported in Table 2.4.3 correspond to values measured at 30 °C which is the most common temperature at which the anammox process is operated in both lab- and full-scale systems, since it is close to the optimal temperature of these organisms (Hu *et al.,* 2013).

As observed in Table 2.4.3, the anammox kinetic rates reported in literature can vary widely. The main reason for such variation appears to be the fraction of active anammox biomass present in the sample. In anammox reactors fed with autotrophic synthetic media, as is often the case for a lab-scale system, a lower fraction of X_{OHO} is expected compared to reactors fed with COD-containing wastewaters. The SRT applied is also expected to affect the fraction of active cells, which may be reduced by the accumulation of a significant fraction of inactive cells and non-biodegradable matter at higher SRT due to decay. Also, certain differences are observed between the kinetics of 1- or 2-stage PN/anammox systems because of the presence of X_{AOO} in the former, which contributes to a reduction in the fraction of active anammox biomass. Finally, since the anammox process is normally operated under nitrite limiting conditions in view of the inhibition potential of this substrate (Lotti *et al.,* 2012), systems where biomass does not tend to aggregate are characterized by lower mass transfer limitations such as flocculent and (especially) suspended sludge, having higher active anammox biomass fractions compared to biofilm systems.

As observed in Table 2.4.3, also the consumption/production stoichiometric ratios may vary for different anammox systems. In literature, there is evidence that the anammox stoichiometry can be affected by the physiological state of the biomass, which can be influenced by the N-load (Dosta *et al.,* 2008; Yang *et al*., 2009), temperature (Dosta *et al.,* 2008) or pH (Carvajal-Arroyo *et al.,* 2013).

2.4.3.9 Type of batch tests

Depending on the type of process of interest (nitrification, denitrification, anammox) and on the selected tracking technique (chemical, titrimetric or manometric), various tests can be performed to assess the nitrogen removal conversions.

A comprehensive list of tests that are described later on in this chapter is presented in Table 2.4.4.

In the following paragraphs, these tests are described in detail. First, nitrification tests are presented (Section 2.4.4), then denitrification tests (Section 2.4.5) and finally anammox tests (Section 2.4.6).

Table 2.4.4 Batch activity tests performed to assess the biological nitrogen removal conversions as a function of the process and tracking method.

Test code	Process	Tracking method	Purpose
NIT.CHE	Nitrification	Chemical	Assessing the maximum NH_4 oxidation rate
NIT.TIT.1	Nitrification	Titrimetric	Assessing the maximum NH_4 oxidation rate
NIT.TIT.2	Nitrification	Titrimetric	Assessing the maximum NH_4 and NO_2 oxidation rate and the ammonification rate
DEN.CHE.1	Denitrification	Chemical	Assessing the maximum denitrification rate and the anoxic growth yield on a specific C source
DEN.CHE.2	Denitrification	Chemical	Assessing the denitrification potential of a wastewater
DEN.MAN	Denitrification	Manometric	Assessing the maximum denitrification rate
DEN.TIT	Denitrification	Titrimetric	Assessing the maximum denitrification rate
AMX.CHE	Anammox	Chemical	Assessing the maximum anammox rate and the NO_2/ NH_4 and NO_3/ NH_4 ratio
AMX.MAN	Anammox	Manometric	Assessing the maximum anammox rate

2.4.4 Nitrification batch activity tests: preparation

2.4.4.1 Apparatus

Each tracking methodology (chemical, titrimetric or manometric) has specific apparatus requirements. When the chemical tracking is applied, refer to the following list of equipment:

1. A batch reactor equipped with mixing system and adequate sampling ports (Section 2.4.3.1).
2. A calibrated pH electrode (if not included/incorporated in the batch reactor setup).
3. A 2-way pH controller for HCl and NaOH addition (alternatively a one-way control - generally for HCl addition - or manual pH control can be applied through the manual addition of HCl and NaOH). For alkaline solutions that needs to be acidified, sparging with gaseous CO_2 (or a gas mixture enriched in CO_2) can be considered instead of the addition of an acidic solution since it has the advantage of avoiding the addition of the counter ions of protons (i.e. Cl^- when using HCl as the acidic solution).
4. A thermometer (with a recommended working temperature range of 0 °C to 40 °C).
5. A temperature control system (if not included in the batch reactor setup).
6. A DO meter with electrode (if not included/incorporated in the batch reactor setup) to verify the aerobic/anoxic conditions.
7. A stopwatch.

A list of the equipment required for titrimetric and manometric tests is described in Section 2.4.3.1.

2.4.4.2 Materials

For general instructions on material preparation, refer to Section 2.2.3.4 and Table 2.2.2. Test-specific requirements will be listed within each protocol of the test. For a complete list of the required materials refer to Section 2.2.3.2 (with the exception of points 7 and 8).

2.4.4.3 Media preparation

- **Real wastewater**

For batch tests that require the use of a real wastewater, follow the instructions reported in Section 2.2.3.3.

- **Titration solutions**

a. NaOH and HCl solutions are needed. Typically 0.05-0.1 N solutions would be suitable for most applications.
b. The H_2O_2 solution can be obtained by dilution of commercially available H_2O_2 solutions. The most common H_2O_2 solution is 3 %, corresponding to a

concentration of 0.44 mol O_2 L^{-1}. Therefore, an appropriate oxygenated titration solution can be obtained by diluting this solution 10 times (a final concentration of 44 mmol O_2 L^{-1}). To check the concentration of the H_2O_2 solution, the iodometric method can be applied (method 4,500-Cl B in APHA *et al.,* 2012). The diluted solution should be stored in dark bottles and new solutions should be prepared every 7-10 days.

- **Ammonium and nitrite stock solutions**

These can be prepared from salts (e.g. from NH_4Cl and $NaNO_2$). An adequate concentration of the stock solutions is between 5 and 10 g N L^{-1}. The pH of the ammonium solution should be adjusted to 7.0 to reduce any potential interference during the pH-static tests.

- **Allyl-N-thiourea (ATU)**

A stock solution of approximately 5-10 g L^{-1} is generally adequate.

- **Acid and base solutions**

These should be 100-250 mL of 0.2 M HCl and 100-250 mL 0.2 M NaOH solutions for automatic or manual pH control, and 10-50 mL of 1 M HCl and 10-50 mL 1 M NaOH solutions for initial pH adjustment if the desired operational pH is very different from the pKa value of the buffering agent. Instead of NaOH, 0.2 M Na_2CO_3 can be used as the alkaline solution, which has the advantage of acting as both the base and carbon source. The use of Na_2CO_3 as the base solution is therefore recommended when the wastewater may have a deficiency of inorganic carbon. This paragraph does not apply when titrimetry is used.

- **Synthetic medium**

This should contain all the required macro- (sodium, chloride, phosphate, magnesium, sulphate, calcium, potassium) and micro-nutrients (iron, zinc, copper, manganese, boron, molybdate, cobalt iodide) to ensure that cells are not limited and in extreme cases to avoid the failure of the test. Thus, although their concentrations may seem very low, one must make sure that all of the constituents are added to the solution in the required amounts. Regarding the macro-nutrients, the following composition (amounts per litre of the nutrient solution) is recommended (based on Kampschreur *et al*., 2007): 72 mg NaH_2PO_4, 35 mg $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot 2H_2O$, 180 mg NaCl, 30 mg KCl, and 1 mg yeast extract. Micro-nutrients can be supplied by dosing 0.3 mL L^{-1} of a trace element solution containing (per litre of solution, recipe based on Kampschreur *et al*., 2007): 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.18 g KI, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.15 g $CoCl_2 \cdot 6H_2O$. Other similar nutrient solutions can be used as long as they contain all the previously reported required nutrients.

- **Washing media**

If the sludge sample must be washed to remove an undesirable compound (which may be even inhibitory or toxic), a washing media should be prepared. The same synthetic medium described above can be used as a washing medium. The washing process can be repeated twice or three times applying the procedure described in Section 2.2.3.5. Thereafter, the following preparation steps of the batch activity tests can be performed. In special cases when sludge from a full-scale plant is used, the plant effluent may be used for washing purposes (only if it does not contain toxic or inhibitory compounds).

Prior to the execution of the experiment, samples of the media and mixed liquor or activated sludge used to perform the tests should be collected to confirm/check the initial (desired) concentration of parameter(s) of interest (e.g. ammonium, nitrite, nitrate, X_{TSS}, X_{VSS}).

Finally, the required working and stock solutions to carry out the determination of the analytical parameters of interest must also be prepared in accordance with Standard Methods (APHA *et al.,* 2012) and the corresponding protocols.

2.4.5 Nitrification batch activity tests: execution

Test NIT.CHE Nitrification chemical test: assessing the maximum ammonium oxidation rate

Activated sludge preparation

1. For sample collection please refer to Section 2.4.3.4.
2. For conventional activated sludge samples from wastewater treatment plants treating urban wastewaters, a sample with a X_{VSS} concentration of around 2-4 g VSS L^{-1} would be suitable. X_{VSS} adjustment can be performed as suggested in Section 2.4.3.5.
3. Pour a defined volume (V_{ML}) of a mixed liquor sample (typically 1 to 3 L) into the reaction vessel and start mixing, aeration and the temperature and pH control systems to maintain temperature and pH around the desired set point values. Select a desired set point pH value; typically values between 7.5 and

8.4 are adequate. If an automated pH control is not available, correct the pH to the desired value by manual addition of an acid/base solution. In principle, the closer the pH set point value to the typical operational pH at the plant, the closer and more representative the nitrification rate will be. The same principle applies to the selection of the temperature set point value. As for DO, the aeration system should provide sufficient oxygen to avoid DO-limiting conditions during the execution of the nitrification tests. This means that under endogenous conditions the observed DO value should be high (e.g. > 6 mg L^{-1}).

4. Wait for approximately 30 min to reach and ensure stable initial conditions. This pre-incubation phase will normally allow any residual nitrite remaining from the plant or source of origin of the sludge to be consumed.

Execution of the test

1. Verify that the temperature, pH, and DO readings are at the desired set point values or at least within the selected intervals. Otherwise adjust the operating conditions accordingly and wait until the system stabilizes.
2. Once stable conditions are reached, add the ammonium solution (having previously adjusted its temperature to the target temperature of the test) to achieve a neither limiting nor inhibiting ammonium concentration in the mixed liquor. Typical and adequate values are between 20 and 40 mg N L^{-1}.
3. Start the stopwatch to keep precise track of the sampling times, since formally the test starts with the addition of the ammonium solution. Collect the activated sludge samples every 20-30 min throughout the execution of the test. Note that all samples need to be filtered through 0.45 µm pore size filters (or smaller), except those used for the determination of X_{TSS} and X_{VSS} concentrations.
4. Conclude the test after 3 to 4 h or, if the sampling and analytical determination of ammonium in the collected samples allows it, when ammonium is depleted.
5. After the conclusion of the test, take a sample for the final X_{VSS} assessment.

Note that nitrite is rather unstable; therefore, nitrite concentrations have to be quickly assessed after sampling in the same day (see Table 2.2.2).

Data analysis

A typical output of this test is outlined in Figure 2.4.3. On the y-axis, the ammonium, nitrite and nitrate concentrations (in mg N L^{-1}) are reported, while time (in hours) is reported on the X-axis. The linear regression over these data allows for the assessment of the ammonium removal and nitrate production rates (in mg N L^{-1} h^{-1}). Please note that the collected data should be sufficient to reliably estimate the corresponding removal/production rate (e.g. r_{NH4} and r_{NO3}) by linear regression with a satisfactory coefficient of determination (e.g. $R^2 > 0.98$). Thus, preferably at least 4 to 5 data points are needed to carry out the linear regression, implying that a larger number of samples will need to be collected in the beginning of the test.

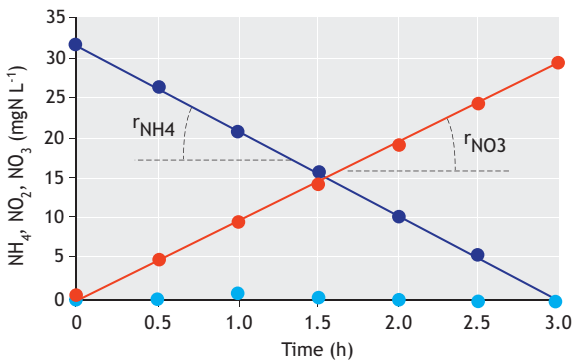

Figure 2.4.3 Typical profiles obtained in a Test NIT.CHE: ammonium (●), nitrite (●) and nitrate (●) concentrations are displayed on the y-axis. Relevant rates of interest are also displayed (e.g. ammonium removal rate r_{NH4}, and nitrate production rate r_{NO3}).

Nitrite may accumulate up to few mg N L^{-1} when conventional activated sludge is used. However, if the activated sludge is used to perform (partial) nitritation tests, then a higher nitrite accumulation will be expected and its concentration should be monitored in time, similar to ammonium and nitrate (for example see Test NIT.TIT.2). When using conventional activated sludge to perform the full oxidation of ammonium to nitrate, the ammonium removal rate should equal the nitrate production rate with a negligible accumulation of nitrite during the test. The maximum specific ammonium oxidation rate ($q_{AOO,NH4}$, as mg N g VSS^{-1} h^{-1}) can be therefore computed as follows:

$$q_{AOO,NH4} = r_{NH4} / X_{VSS} \qquad \text{Eq. 2.4.24}$$

Test NIT.TIT.1 Nitrification titration test: assessing the maximum ammonium oxidation rate

Activated sludge preparation

1. For conventional activated sludge samples from wastewater treatment plants treating urban wastewaters, a sample with a X_{VSS} of around 2-4 g VSS L^{-1} will be suitable. For very diluted or concentrated sludge samples, a concentration step (e.g. by decanting into an Imhoff cone for 30 min or by centrifuging at 4,000 rpm for a few minutes) or dilution with secondary effluent from the same wastewater treatment plant may be helpful. This will avoid having too slow or too fast nitrification rates.
2. Pour a known volume of the activated sludge sample (typically 1 L) into the reaction vessel and activate the aeration and the temperature control systems. Select a desired set point pH value. Typically, values between 7.5 and 8.4 are adequate. In principle, the closer the set point pH value to the typical operational pH of the plant or source of sludge, the closer and more representative the observed nitrification rate will be. The same principle applies to the selection of the temperature value. Concerning DO, the aeration system should provide sufficient oxygen to avoid DO-limiting conditions during the course of the nitrification tests. This means that under endogenous conditions the observed DO value should be relatively high (e.g. > 6 mg L^{-1}).
3. Activate the automated titration system for a pre-incubation period of approximately 1 h. This pre-incubation phase will ensure that: (*i*) endogenous conditions are achieved at the start of the titration test (S_B, ammonium and nitrite are oxidized during this overnight aeration phase) and (*ii*) that temperature, pH and S_{O2} are stable at the start of the test. Pre-humidified air may be used to limit significant water evaporation during this pre-incubation phase. Note that prolonged incubation periods (e.g. longer than 4 h) may reduce the nitrification rate due to fast endogenous biomass decay under aerobic conditions.

Execution of the test

1. Activate the data logging.
2. Add the ammonium chloride stock solution (having previously adjusted its temperature to the target temperature of the test) to achieve an ammonium concentration in the activated sludge that is neither limiting nor inhibiting (between 20 and 40 mg N L^{-1} are typically adequate values). NH_4Cl addition to an alkaline suspension has an acidifying affect (acid hydrolysis) that leads to a rapid pH drop that should be compensated by an automatic pH control system or through the manual addition of concentrated NaOH. Any addition of the titration solutions during this pH-adjustment phase should be disregarded during the data analysis. Upon the ammonium addition, nitrifying bacteria will oxidize the ammonium added and consequently an alkaline titrating solution will need to be added to compensate for the acidifying nitrification effect.
3. Record the volume of the NaOH titration solution added over time (V_{NaOH} versus time) (20-40 min are usually adequate). Check that the pH reading value remains close to the target pH set point ± 0.02 and that the S_{O2} level does not become limiting. Do not change the aeration rate or the mixing conditions since this would affect the titration rate, making the assessment of the nitrification-related titration rate cumbersome. The collected data should be sufficient to reliably estimate the titration rate (Q_{NaOH}) from a linear regression of V_{NaOH} versus time data with a satisfactory coefficient of determination ($R^2 > 0.98$).
4. Add allyl-N-thiourea in order to achieve a final ATU concentration of 10 mg L^{-1}. At this concentration, ammonium oxidation will be inhibited. Continue recording the NaOH titration rate for a further 20-30 min in order to assess the residual titration rate due to background pH affection reactions such as CO_2 stripping ($Q_{NaOH,final}$), if present. Note that the longer the pre-incubation period the lower the relevance of $Q_{NaOH,final}$.
5. The test can now be ended. Measure the final activated sludge volume and take a sample to assess the X_{VSS} concentration. Note that the suspension volume will change during the course of the test due to the addition of titration solutions. It is expected that the addition of titration solution does not account for more than 10 % of the final activated sludge volume.

Data analysis

A typical trend of the volume of NaOH added during the test is depicted in Figure 2.4.4. From these data (titration volume versus time data), titration rates (Q) can be computed through the slope of the titration curve using the corresponding tool in a worksheet or by applying the following formula:

$$Q = \frac{n \cdot \sum t_i \cdot V_{NaOH,i} - \sum t_i \cdot \sum V_{NaOH,i}}{n \cdot \sum t_i^2 - (\sum t_i)^2} \qquad \text{Eq. 2.4.25}$$

Where, n is the number of recorded data [t_i, $V_{NaOH,i}$] available.

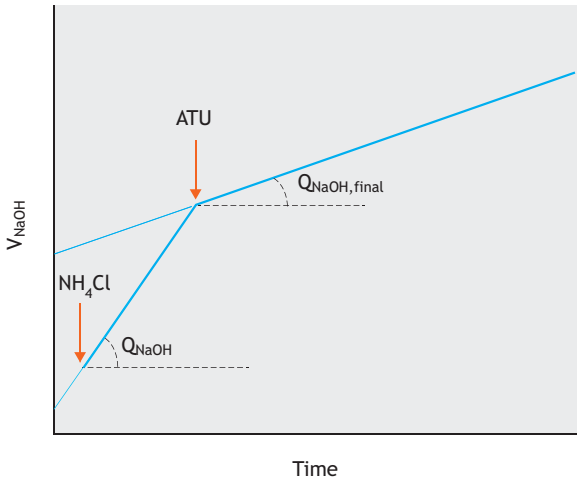

Figure 2.4.4 Example of a pH-static titration curve during a nitrification test to assess the maximum ammonium-oxidizing capacity. Arrows indicate the addition of ammonium chloride and allyl-N-thiourea. Relevant titration rates are also identified on the graph.

The NaOH titration rates (Q_{NaOH} and $Q_{NaOH,final}$ in mL min^{-1}) are used to assess the ammonium oxidation rate (F_{NHx} in mg N min^{-1}) by taking into account the concentration of the NaOH solution (N_{NaOH} in meq mL^{-1}) and the ratio between ammonium oxidation and alkalinity consumption Y_{NH4_H+} that can be assessed from Eq. 2.4.15. Therefore:

$$F_{NHx} = (Q_{NaOH} - Q_{NaOH,final}) \cdot N_{NaOH} \cdot Y_{NH4\_H+} \quad \text{Eq. 2.4.26}$$

Finally, the maximum specific ammonium oxidation rate of the sludge ($q_{AOO,NH4}$, in mg N g VSS^{-1} h^{-1}) can be computed by taking into account the X_{VSS} concentration of the sludge sample (in g VSS L^{-1}) and the suspension volume observed at the end of the test (V_{ML}):

$$q_{AOO,NH4} = 60 \cdot F_{NHx} / (V_{ML} \cdot X_{VSS}) \quad \text{Eq. 2.4.27}$$

Test NIT.TIT.2 Nitrification titration test: assessing the maximum ammonium and nitrite oxidation rates

Activated sludge preparation

Follow steps 1, 2 and 3 of the activated sludge preparation described for Test NIT.TIT.1.

Execution of the test

1. Select an appropriate set point value for S_{O2} (DO set point). Typically values between 4.0 mg N L^{-1} and 6.0 mg N L^{-1} are adequate to assess the maximum nitrification rates. Activate the data-logging system.
2. Record the volumes of the H_2O_2 and NaOH titration solutions added over time (V_{H2O2} and V_{NaOH} versus time) (20-40 min are normally adequate). Check that the pH and DO values remain within the interval pH set point ± 0.02 and S_{O2} set point ± 0.10 mg L^{-1}, respectively. The collected data should be sufficient to reliably estimate (i.e. with a satisfactory coefficient of determination, $R^2 > 0.98$) the alkaline titration rate from the linear regression of V_{NaOH} versus time data, and the oxygen titration rate (Q_{H2O2}) from the linear regression of V_{H2O2} versus time data. During this phase, titration rates are triggered by endogenous respiration which leads to DO consumption and CO_2 production; the former is compensated by H_2O_2 addition (at a rate indicated as $Q_{H2O2,ini}$), and the latter by NaOH addition (at a rate indicated as $Q_{NaOH,ini}$).
3. Add nitrite at a neither limiting nor inhibiting nitrite concentration in the activated sludge (around 10 mg N L^{-1} are typically adequate values) in order to trigger nitrite oxidation. Repeat data acquisition as described in Step 2 in order to estimate the oxygen titration rate that includes the oxygen request for nitrite oxidation ($Q_{H2O2,NO2}$). The alkaline titration rate will not change since nitrite oxidation does not significantly affect the suspension pH.
4. Add the ammonium chloride stock solution according to the instructions reported in step 2 of the test operation procedure described for Test NIT.TIT.1. This addition will trigger ammonium oxidation as well. Repeat data acquisition as described in step 2 to assess the alkaline titration rate ($Q_{NaOH,NH4}$) and the oxygen titration rate ($Q_{H2O2,NH4}$) that include the ammonium oxidation needs.
5. Add allyl-N-thiourea (ATU) to a final concentration of 10 mg L^{-1}. Ammonium oxidation will be inhibited. Continue recording the NaOH titration rate for a further 20-30 min to assess the residual titration rate due to the background pH affecting reactions such as CO_2 production ($Q_{NaOH,final}$) and oxygen-affecting reactions ($Q_{H2O2,final}$) including endogenous respiration and residual nitrite oxidation.
6. End the test according to the instructions reported in Step 4 of the test operation procedure described for Test NIT.TIT.1.

Data analysis

A typical trend of the cumulated volume of titration solutions added during the test is depicted in Figure 2.4.5. From these data (volume versus time data), the titration

rates (Q) can be computed as the slope of the titration curve using the corresponding tool in a worksheet or by applying the formula previously described in Test NIT.TIT.1.

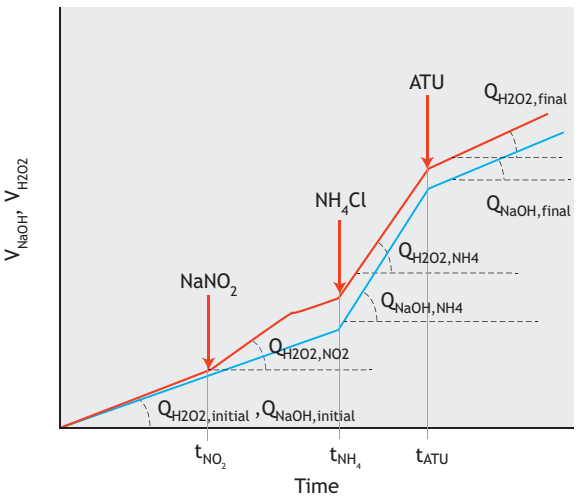

Figure 2.4.5 Example of a pH/DO-stat titration curve during a nitrification test executed to assess the maximum ammonium- and nitrite-oxidizing capacity. Arrows indicate the addition of nitrite, allyl-N-thiourea and ammonium. Relevant titration rates of interest are also displayed on the graph.

The NaOH titration rates ($Q_{NaOH,NH4}$ and $Q_{NaOH,final}$ in mL min^{-1}) can be first used to assess the ammonium oxidation rate ($F_{NHx,NaOH}$ in mg N min^{-1}) by taking into account the concentration of the NaOH titration solutions (N_{NaOH} in meq mL^{-1}) and the ratio between ammonium oxidation and alkalinity consumption, as suggested in Test NIT.TIT.1:

$$F_{NHx,NaOH} = (Q_{NaOH,\,NH4} - Q_{NaOH,\,final}) \cdot N_{NaOH} \cdot Y_{NH4\_H+} \quad \text{Eq. 2.4.28}$$

Similarly, oxygen titration rates ($Q_{H2O2,NH4}$ and $Q_{H2O2,final}$ in mL min^{-1}) can be first used to assess the ammonium oxidation rate ($F_{NHx,H2O2}$ in mg N min^{-1}) by taking into account the concentration of the H_2O_2 titration solutions (N_{H2O2} in mmol O_2 mL^{-1}) and the ratio between ammonium oxidation to nitrate and oxygen consumption, $Y_{NH4/O2\_NO3}$, that is, according to the ASM nitrification stoichiometry (Henze *et al.,* 2000):

$$F_{NHx,\,H2O2} = (Q_{H2O2,NH4} - Q_{H2O2,final}) \cdot N_{H2O2} \cdot 32 \cdot Y_{NH4/O2\_NO3} \quad \text{Eq. 2.4.29}$$

$$Y_{NH4/O2\_NO3} = \frac{1}{4.57 - Y_{AOO}} = 0.23 \text{ g N g } O_2^{-1} \quad \text{Eq. 2.4.30}$$

The values of $F_{NHx,NaOH}$ and $F_{NHx,H2O2}$ should be similar and their comparison can be used to validate the experimental data. Differences higher than 15 % may suggest the need for a careful verification of the experimental setup.

The oxygen titration rates collected during step 3 ($Q_{H2O2,NO2}$ in mL min^{-1}) will be used to determine the nitrite oxidation rate (F_{NO2} in mg N min^{-1}) by taking into account the ratio between nitrite oxidation to nitrate and oxygen consumption, $Y_{NO2/O2\_NO3}$, as follows (according to the two-step nitrification stoichiometry):

$$F_{NO2} = (Q_{H2O2,NO2} - Q_{H2O2,initial}) \cdot N_{H2O2} \cdot 32 \cdot Y_{NO2/O2\_NO3} \quad \text{Eq. 2.4.31}$$

$$Y_{NO2/O2\_NO3} = \frac{1}{1.14} = 0{,}88 \text{ g N g } O_2^{-1} \quad \text{Eq. 2.4.32}$$

If no difference is observed between $Q_{H2O2,NO2}$ and $Q_{H2O2,initial}$ values then either the nitrite oxidation rate is very slow or it is much slower than the ammonium oxidation rate. This may lead to nitrite accumulation during the endogenous phase since ammonium oxidation would probably take place on the ammonium released through ammonification. If so, $Q_{H2O2,initial}$ would include oxygen consumption due to the slow nitrite oxidation process and be equal to $Q_{H2O2,NO2}$. In this case, the activated sludge sample should be elutriated to remove any nitrite content by centrifugation and resuspension in a nitrite-free physiological medium to assess the nitrite oxidation rate. However, when the nitrite oxidation rate is very slow, chemical tracking over a longer testing period (a few hours) may lead to more reliable estimates and should be preferred. Note that, for very slow nitrite oxidation rates, $F_{NHx,H2O2}$ may be higher than $F_{NHx,NaOH}$. As a matter of fact, the use of $Y_{NH4/O2\_NO3}$ is no more correct since the sole ammonium oxidation to nitrite request should be taken into account, since $Y_{NH4/O2\_NO3}$ quantifies the overall ammonium plus nitrite oxidation request. Therefore, in such a case, $F_{NHx,H2O2}$ would be more correctly estimated taking into account the ratio between ammonium oxidized and oxygen consumption for ammonium oxidation to nitrite, $Y_{NH4/O2\_NO2}$, which

can be expressed according to the two-step nitrification stoichiometry expression as:

$$F_{NHx,H2O2} = (Q_{H2O2,NH4} - Q_{H2O2,final}) \cdot N_{H2O2} \cdot 32 \cdot Y_{NO2/O2\_NO2} \quad \text{Eq. 2.4.33}$$

$$Y_{NO2/O2\_NO2} = \frac{1}{3.43 - Y_{AOO}} = 0.31 \text{ g N g } O_2^{-1} \quad \text{Eq. 2.4.34}$$

Moreover, the difference between $Q_{NaOH,initial}$ and $Q_{NaOH,final}$ makes it possible to estimate the ammonification rate under endogenous conditions. The first titration rate compensates for the alkaline effect of endogenous respiration and of nitrification, which is limited by the ammonification process responsible for ammonium release. The ammonification-related alkalizing effect is no longer present after ATU addition. Thus, the ammonification rate (F_{N_NHx} in mg N min^{-1}) can be estimated, by difference, according to the following equation:

$$F_{N\_NHx} = Y_{NH4/O2\_NO3} \cdot (Q_{NaOH,initial} - Q_{NaOH,final}) \cdot N_{NaOH} \quad \text{Eq. 2.4.35}$$

Finally, the maximum biomass-specific ammonium and nitrite oxidation rates of the sludge ($q_{AOO,NHx}$, and $q_{NOO,NO2\_NO3}$ in mg N g VSS^{-1} h^{-1}) and the specific ammonification rate (q_{N_NHx}), can be computed by taking into account the X_{VSS} concentration of the sludge sample (in g VSS L^{-1}) and the suspension volume observed at the end of the test (V_{ML}):

$$q_{AOO,NHx} = 60 \cdot F_{NHx} / (V_{ML} \cdot X_{VSS}) \quad \text{Eq. 2.4.27}$$

$$q_{NOO,NO3\_NO2} = 60 \cdot F_{NO2} / (V_{ML} \cdot X_{VSS}) \quad \text{Eq. 2.4.36}$$

$$q_{N\_NHx} = 60 \cdot F_{N\_NHx} / (V_{ML} \cdot X_{VSS}) \quad \text{Eq. 2.4.37}$$

2.4.6 Denitrification batch activity tests: preparation

These tests are meant to assess the maximum denitrification rate of a sludge sample and the anoxic biomass growth yield. Various types of carbon sources can also be used such as internal carbon, external carbon sources (e.g. sugar or alcohol) or wastewater. Typically, the maximum denitrification rate is expressed when a rapidly biodegradable carbon source (to which the sludge is adapted) is used, while lower rates are observed in the presence of complex organic molecules that require a preliminary hydrolysis step or when dosing external carbon solutions that require specialized metabolic capabilities/microorganisms.

Four tests are presented. The first one (DEN.CHE.1) refers to the use of an easily biodegradable carbon source, for which both the denitrification rate and the biomass anoxic growth yield are relevant parameters to be assessed. In the second test (DEN.CHE.2), a real wastewater is used. Although kinetic information can be drawn from this, this second test is mainly meant to assess the denitrification capacity of this wastewater, i.e. the amount of nitrate that can be denitrified per unit volume of this specific wastewater. Finally, two more tests are presented for the assessment of the maximum denitrification rate by applying a manometric (DEN.MAN) or titrimetric (DEN.TIT) tracking procedure.

2.4.6.1 Apparatus

Each tracking methodology (chemical, titrimetric or manometric) has special apparatus requirements.

When the chemical tracking is applied refer to the following list of equipment:

1. An (airtight) batch reactor equipped with mixing system and adequate sampling ports (as described in Section 2.4.3.1).
2. A nitrogen gas supply (recommended).
3. A calibrated pH electrode (if not included or incorporated in the batch reactor setup).
4. A 2-way pH controller for HCl and NaOH addition (alternatively a one-way control, generally for HCl addition, or a manual pH control can be applied through the manual addition of HCl and NaOH).
5. A thermometer (with a recommended working temperature range of 0 to 40 °C).
6. A temperature control system (if not included in the batch reactor setup).
7. A DO meter with an electrode (if not included/incorporated in the batch reactor setup) to verify anoxic conditions.
8. A stopwatch.

When titrimetric tests are performed, refer to the following list of equipment:

1. The equipment for the titrimetric system described in Section 2.4.3.1.
2. A nitrogen gas supply (recommended).

When manometric tests are performed, refer to the following list of equipment:

1. The equipment for the manometric system described in Section 2.4.3.1.
2. A nitrogen gas supply (recommended).

2.4.6.2 Materials

For a complete list of required materials refer to Section 2.2.3.2 (with the exception of points 7 and 8).

2.4.6.3 Working solutions

- **Real wastewater**

For batch tests that require the use of real wastewater, follow the instructions given in Section 2.2.3.3.

- **Carbon source solution**

This is usually composed of a readily biodegradable carbon source (S_B), preferably volatile fatty acids like acetate or propionate, sugars, or alcohol solutions. The choice of the organic carbon source depends on the nature or goal of the test and the corresponding research questions. Sometimes more complex substrates are used that are more similar to real wastewaters, containing a mixture of readily and slowly biodegradable COD. For anoxic batch activity tests, the COD concentration (both total and soluble) should be known in order to select a proper dose.

- **Nitrate or nitrite solutions**

Nitrate and nitrite salts are needed to adjust the nitrate/nitrite level during denitrification tests.

- **Washing media**

If the sludge sample must be 'washed' to remove undesirable compounds, then refer to Section 2.4.3.4.

- **Acid and base solutions**

These are 100-250 mL of 0.2 M HCl and 100-250 mL 0.2 M NaOH solutions for automatic or manual pH control, and 10-50 mL of 1 M HCl and 10-50 mL 1 M NaOH solutions for an initial pH adjustment if the desired operational pH is very different from the pKa value of the buffering agent. For titrimetric tests, titration solutions should be prepared according to the instructions given in Section 2.4.4.3.

- **Nutrient solution**

This should contain all the required macro- (ammonium, magnesium, sulphate, calcium, potassium) and micro-nutrients (iron, zinc, calcium, copper, manganese, molybdate, cobalt) to ensure that cells are not short of basic nutrients for their metabolism. Thus, despite the fact that their concentrations may seem very low, one must make sure that all of the constituents are added to the solution in the required amounts. Regarding macro-nutrients the following composition (amounts per litre of nutrient solution) is recommended (based on Smolders *et al*., 1994): 107 mg NH_4Cl, 90 mg $MgSO_4 \cdot 7H2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract. Micro-nutrients can be supplied by dosing 10 mL L^{-1} of a trace element solution containing (per litre of solution) (based on Vishniac and Santer, 1957): 50 g EDTA, 22 g $ZnSO_4 \cdot 7H_2O$, 5.54 g $CaCl_2$, 5.06 g $MnCl_2 \cdot 4H_2O$, 4.99 g $FeSO_4 \cdot 7H_2O$, 1.10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.57 g $CuSO_4 \cdot 5H_2O$, and, 1.61 g $CoCl_2 \cdot 6H_2O$. Similar nutrient solutions can be used as long as they contain all the previously reported required nutrients.

It is recommended to take a sample of the media and sludge prior to the execution of the experiment to confirm/check the initial (desired) concentration of parameter(s) of interest (e.g. COD, nitrate/nitrate, X_{VSS}).

Finally, the required working and stock solutions to carry out the determination of the analytical parameters of interest must be also prepared in accordance to Standard Methods (APHA *et al.,* 2012) and the corresponding protocols for their preservation and analytical determination.

2.4.6.4 Material preparation

For general instructions on how to organize the material preparation, please refer to Section 2.2.3.4 and to Table 2.2.2. Test-specific requirements will be listed within each test protocol.

2.4.7 Denitrification batch activity tests: execution

Test DEN.CHE.1 Denitrification chemical test: assessing the maximum denitrification rate and the anoxic growth yield in the presence of a specific carbon source

Activated sludge preparation

The optimal sampling point for the activated sludge would be the outlet of the post-denitrification tank.

1. For conventional activated sludge samples treating urban wastewaters, a sample with a X_{VSS} of around 2-4 g VSS L^{-1} would be suitable. The X_{VSS} can be adjusted as suggested in Section 2.4.3.5.
2. Pour a known volume (V_{ML}) of activated sludge sample (typically 1 to 3 L) into the reaction vessel and

start mixing. Also start the temperature and pH control systems to keep both parameters around the desired set point values.

3. Sparge N_2 into the reaction headspace for approximately 10 min to ensure a deoxygenated environment. Ensure a gas outlet to limit overpressure. Gas sparging can be continued until the end of the test. If this is not feasible, then a proper airtight reactor with a gas outlet preventing oxygen back-diffusion (e.g. by using a unidirectional check valve or a water lock) should be used (see Section 2.2.2.1 for more information on how to ensure anoxic conditions).
4. Wait for approximately 30 min to ensure stable initial conditions. This pre-incubation phase will usually allow the removal of any residual nitrate.

Execution of the test

Verify that your target values of temperature, pH, and S_{O2} are close to the set points or within the selected intervals. Otherwise adjust and wait until the system stabilizes.

1. Add the nitrate and S_B stock solutions. The temperature of both these solutions should have been previously adjusted to the target temperature of interest. The initial nitrate concentration should be neither limiting nor inhibitory (20 to 25 mg N L^{-1} are typically adequate values). If residual nitrate is expected to be present in the activated sludge sample, the nitrate addition should then be reduced.
2. Add a non-limiting amount of the readily biodegradable carbon source (S_B). To assess the appropriate amount of S_B, one can consider the stoichiometric relationship between the amount of S_B and nitrate consumed during denitrification:

$$Y_{NO3\_SB,Ax} = \frac{2.86}{1 - Y_{OHO,Ax}} \text{ (g COD g } N^{-1}) \qquad \text{Eq. 2.4.38}$$

3. The S_B addition should guarantee that the S_B to nitrate ratio should be at least twice as much as the stoichiometric value found with the previous expression. Note that the anoxic biomass growth yield, $Y_{OHO,Ax}$, depends on the carbon source, as reported in Table 2.4.2. However, a value of 0.5 can be typically used for a rough estimation. Under this assumption, a S_B concentration in the activated sludge of 200 mg COD L^{-1} would usually be adequate. This concentration also satisfies the initial S_B to X_{VSS} ratio value (0.05-0.1 g COD g VSS^{-1}) suggested in Section 2.2.4.1. Lower values may result in too rapid carbon depletion while too high values may lead to biomass inhibition. An ammonium stock solution can also be added to adjust the ammonium to S_B ratio to 0.05 g N g COD^{-1}.
4. The test starts with the addition of the nitrate and S_B solutions. Start the stopwatch to keep precise track of the following sampling times and start the sampling campaign.
5. Collect activated sludge samples at regular time intervals. As a general suggestion, samples for the determination of the C source (or of soluble COD depending upon the analytical parameter of interest) and of nitrite and nitrate must be collected every 10 min in the first 30 min after S_B addition, every 15 min during the following 60 min, and later on every 30 min until the end of the test.
6. Conclude the test when the nitrate and nitrite are fully depleted. Use nitrate and nitrite strip tests to quickly assess when these compounds have been consumed.
7. Take a sample for final X_{VSS} concentration assessment.

It should be noted that nitrite concentration is unstable, and therefore nitrate and nitrite concentrations have to be quickly measured, preferably on the same day.

Data analysis

The typical output of this test is outlined in Figure 2.4.6.

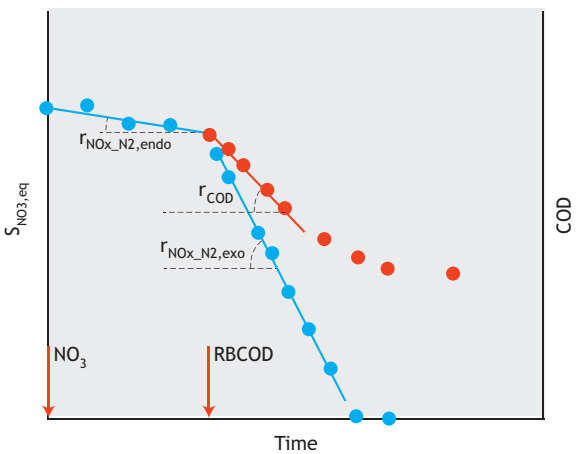

Figure 2.4.6 Typical profiles obtained in a Test DEN.CHE.1: nitrate concentrations (●) on the main y-axis, COD concentrations (●) on the secondary y-axis. Relevant rates of interest are also displayed (endogenous denitrification rate $r_{NOx\_N2,endo}$, exogenous denitrification rate, $r_{NOx\_N2,exo}$, and COD consumption rate r_{COD}). The arrow indicates the substrate addition.

On the principal y-axis, the oxidized nitrogen equivalent $S_{NO3,Eq}$ is reported, which corresponds to a weighted sum of the nitrate and nitrite concentrations:

$$S_{NO3,Eq} = S_{NO3} + 0.6 \cdot S_{NO2} \quad \text{Eq. 2.4.39}$$

The 0.6 weight applied to the nitrite concentration corresponds to the relative electron-accepting capacity of nitrite with respect to nitrate (1.71/ 2.86 = 0.6), as suggested by Kujawa and Klapwijk (1999). On the secondary y-axis, soluble COD data are presented. During the first period (i.e. before the addition of the external carbon source), endogenous denitrification takes place and a slow-rate reduction of $S_{NO3,Eq}$ is observed.

The linear regression over these data (see Section 2.4.4.2) can be used to assess the endogenous denitrification rate ($r_{NOx\_N2,endo}$, in mg N L^{-1} min^{-1}). After the S_B addition, the availability of the exogenous carbon source speeds up the consumption of nitrate and nitrite. Collecting the nitrate/nitrite data afterwards, but before nitrate/nitrite becomes limiting, makes it possible to assess the exogenous denitrification rate ($r_{NOx\_N2,exo}$, in mg N L^{-1} min^{-1}). Similarly, the maximum COD consumption rate (r_{COD}, in mg COD L^{-1} min^{-1}) can be assessed within the same timeframe.

The maximum specific denitrification rate on S_B ($q_{NOx\_N2,SB}$ in mg N g VSS^{-1} h^{-1}) on the tested carbon source can be computed as follows:

$$q_{NOx\_N2,SB} = 60 \cdot (r_{NOx\_N2,exo} - r_{NOx\_N2,endo}) / X_{VSS} \quad \text{Eq. 2.4.40}$$

Moreover, by combining denitrification rates and the COD consumption rate, the biomass growth yield ($Y_{OHO,Ax}$) can also be assessed according to the following formula:

$$Y_{OHO,Ax} = 1 - 2.86 \frac{(r_{NOx\_N2,exo} - r_{NOx\_N2,endo})}{r_{COD}} \quad \text{Eq. 2.4.41}$$

Test DEN.CHE.2 Denitrification chemical test: assessing the denitrification potential of wastewater

Activated sludge preparation

Samples for this test should be collected at the outlet of the pre-denitrification tank.

1. Follow step 1 of the activated sludge preparation procedure described for Test DEN.CHE.1 Note that in this case a biomass concentration of 3-4 g VSS L^{-1} would be more adequate since a dilution effect is obtained when the wastewater is added.
2. Pour a known volume of the activated sludge sample (V_{ML}, typically 0.6-0.8 L) and keep a sample of it to assess the volatile suspended solids concentration (MLVSS in g VSS L^{-1}). Follow steps 2, 3 and 4 of the activated sludge preparation procedure described for Test DEN.CHE.1.

Test execution

1. Verify that your target values of temperature, pH, and DO are within the selected intervals. Otherwise adjust them and wait for their stabilization.
2. Select the appropriate volume of wastewater (V_{WW}) to be added. It would be ideal to add an amount of wastewater so that the final biodegradable COD concentration in the reaction vessel remains within 30 and 70 mg L^{-1}. By assuming a typical concentration of biodegradable carbon ($S_B + XC_B$) of 100-180 mg L^{-1}, a dilution factor (V_{ML}:V_{WW}) of 2 to 6 should be appropriate. Pour the wastewater into the reaction vessel and add the nitrate stock solution in order to achieve an initial nitrate concentration in the final mixture (V_{ML}+V_{WW}) of 20-25 mg N L^{-1}.
3. Start a stopwatch in order to keep precise track of the following sampling times and start the sampling campaign. As a general suggestion, samples for the determination of nitrite and nitrate concentration must be collected every 5 min during the first 30-45 min of execution of the test, every 10 or 15 for a further 30-45 min, and later on every 15 or 30 min until the end of the test.
4. Conclude the test after 3-4 h when a slow (endogenous-like) rate is observed.

Data analysis

The typical output of this test is outlined in Figure 2.4.7. On the y-axis, the oxidized nitrogen equivalent $S_{NO3,Eq}$ during the course of the test is given. Various denitrification rates can be observed. The highest rate ($r_{NOx\_N2,SB}$) is observed initially, i.e. when both S_B and XC_B are available to the heterotrophic microorganisms (interval Δt_1 in the graph). Once the S_B organic fraction is fully utilized, denitrification proceeds only on the soluble organics that are made available through the hydrolysis of XC_B, therefore on the so-called slowly biodegradable organic material (interval Δt_2 in the graph). The denitrification rate ($r_{NOx\_N2,XCB}$) is therefore limited by the hydrolysis rate of XC_B. When hydrolysable organics are fully consumed, denitrification can continue on the endogenous carbon at a slower rate ($r_{NOx\_N2,endo}$) as long as nitrate and nitrite are still available.

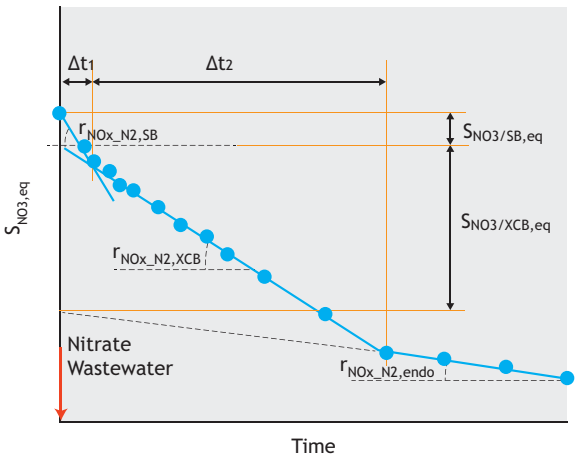

Figure 2.4.7 Typical profiles obtained in a DEN.CHE.2 test. Nitrate concentrations during the execution of the test are displayed ( ). Relevant rates of interest are shown (denitrification rate on the rapidly biodegradable $r_{NOx\_N2,SB}$, and slowly biodegradable $r_{NOx\_N2,XCB}$, fractions and endogenous denitrification rate $r_{NOx\_N2,endo}$). Arrows indicate the nitrate and wastewater additions. Relevant nitrate equivalent variations are also identified ($S_{NO3/SB,eq}$ on S_B and $S_{NO3/XCB,eq}$ on XC_N).

The linear regression of $S_{NO3,Eq}$ versus time data for each interval (see Section 2.4.4.2) allows each relevant denitrification rate (in mg N L^{-1} min^{-1}) to be calculated. Specific denitrification rates (in mg N g VSS^{-1} h^{-1}) can be computed as follows:

- specific denitrification rate on S_B:

$$q_{NOx\_N2,SB} = 60 \cdot (r_{NOx\_N2,SB} - r_{NOx\_N2,endo}) / X_{VSS} \quad \text{Eq. 2.4.42}$$

- specific denitrification rate on XC_B:

$$q_{NOx\_N2,XCB} = 60 \cdot (r_{NOx\_N2,XCB} - r_{NOx\_N2,endo}) / X_{VSS} \quad \text{Eq. 2.4.43}$$

- specific endogenous denitrification rate:

$$q_{NOx\_N2,endo} = 60 \cdot (r_{NOx\_N2,endo}) / X_{VSS} \quad \text{Eq. 2.4.44}$$

The amount of nitrate equivalents that are consumed on the rapidly ($S_{NO3/SB,eq}$) and the slowly ($S_{NO3/XCB,eq}$) biodegradable carbon sources are also illustrated in the graph which can be estimated as follows:

$$S_{NO3/SB,eq} = (r_{NOx\_N2,SB} - r_{NOx\_N2,XCB}) \cdot \Delta t_1 \quad \text{Eq. 2.4.45}$$

$$S_{NO3/XCB,eq} = (r_{NOx\_N2,XCB} - r_{NOx\_N2,endo}) \cdot \Delta t_2 \quad \text{Eq. 2.4.46}$$

By considering the volume of wastewater tested, the denitrification potential of the rapidly biodegradable (DP_{SB}) and slowly biodegradable (DP_{XCB}) organic compounds can be finally assessed:

$$DP_{SB} = \frac{S_{NO3/SB,eq} \cdot (V_{ML} + V_{WW})}{V_{WW}} \quad \text{Eq. 2.4.47}$$

$$DP_{XCB} = \frac{S_{NO3/XCB,eq} \cdot (V_{ML} + V_{WW})}{V_{WW}} \quad \text{Eq. 2.4.48}$$

Test DEN.MAN Denitrification manometric test: assessing the denitrification kinetic rate

Activated sludge preparation

1. Follow step 1 of the activated sludge preparation protocol described for Test DEN.CHE.1.
2. Select the appropriate amount of activated sludge to be poured into the reaction vessel (V_{ML}). For this purpose, one should consider that the extent of the overpressure caused by gas release depends on the remaining headspace volume (V_{HS}) and on the expected N_2 generation, (amount of nitrogen to be denitrified) and the headspace volume (V_{HS}). Hence, the correct selection of this ratio is crucial to avoid extreme pressures (either too high or too low). The maximum overpressure will be achieved at the end of the test, i.e. when all the nitrate has been denitrified, described as:

$$P_{max} - P_{atm} = \frac{P_{atm}}{V_{HS}} \cdot \frac{M_{N2}}{28 \cdot 1{,}000} \cdot 22.4 \cdot \frac{(273 + T_C)}{273} \quad \text{Eq. 2.4.49}$$

Where, T_C is the temperature (°C), M_{N2} is the mass of nitrogen gas generated by denitrification during the course of the test (mg N), which depends on the nitrate concentration ($S_{NO3\_N2,Ax}$, in mg N L^{-1}) and the activated sludge volume, as follows:

$$M_{N2} = S_{NO3\_N2,Ax} \cdot V_{ML} \quad \text{Eq. 2.4.50}$$

By substituting this equation into the previous one and rearranging, the following relationship is obtained:

$$\frac{V_{ML}}{V_{HS}} = \frac{P_{max} - P_{atm}}{P_{atm}} \cdot \frac{28}{22.4} \cdot \frac{273}{(273 + T_C)} \cdot \frac{1}{\frac{S_{NO3\_N2,Ax}}{1{,}000}} \quad \text{Eq. 2.4.51}$$

The optimal value for P_{max} is normally around 0.2 atm, which means that, under typical conditions (T_C

~20 °C, $S_{NO3\_N2,Ax}$ ~20 mg N L^{-1}), a V_{ML} to V_{HS} ratio of around 11 is obtained. Therefore, for a total reactor volume of 1 L, the ideal activated sludge volume to be used during the test will be 0.92 L.

3. Pour the previously computed amount of activated sludge into the reaction vessel. Insert the magnet for the mixing. Insert NaOH pellets in the headspace for CO_2 adsorption. Flush the reactor headspace with N_2 and seal the bottle gas tightly. Place the reactor into the thermostatic chamber and under gentle mixing, wait for 30 min until the temperature stabilizes.

Test execution

1. Determine the volume of nitrate solution to be added (as a pulse or spike) to achieve a nitrate concentration in the activated sludge of 20-25 mg N L^{-1}. Using a syringe, inject the nitrate stock solution through the rubber septum. Select the amount of carbon source to be dosed (also as a pulse or spike) in order to operate under non-limiting and non-inhibiting conditions (see step 4 of the execution of Test DEN.CHE). Then inject the carbon source solution and follow up the time execution with a stopwatch.
2. Start the manometric data collection. Collect data every 15-30 min or until a bending point is observed in the overpressure curve, which indicates the exhaustion of nitrate.
3. End the test. Check the final pH and take a final sample to measure the MLVSS concentration.

Data analysis

A typical output of a manometric denitrification test using a manometer with a data logger is outlined in Figure 2.4.8.

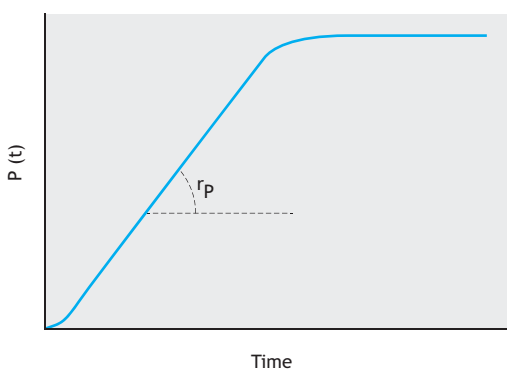

Figure 2.4.8 Typical overpressure profile obtained in a manometric denitrification test performed with a manometer equipped with a data logger. The chart displays the maximum pressure production rate r_P.

During the first 10-15 min, various phenomena may overlap caused by potential interferences such as: (*i*) intrusion into the bulk liquid of residual oxygen that may remain in the headspace, (*ii*) water vapour pressure equilibrium. (*iii*) initial N_2 accumulation in the liquid phase, and/or, (*iv*) microbiological lag phases. For this reason, the initial overpressure data should be disregarded. The following overpressure data can be used to compute the pressure production rate (r_P, atm min^{-1}) by linear regression (see Section 2.4.3.3).

From r_P, the denitrification rate (F_{NO3_N2}, mg N min^{-1}) can be assessed as follows:

$$F_{NO3\_N2} = \frac{r_p}{P_{atm}} \cdot V_{HS} \cdot \frac{28}{22.4} \cdot \frac{273}{273 + T_C} \qquad \text{Eq. 2.4.52}$$

With, P_{atm} in atm and V_{HS} in mL.

Finally, the specific denitrification rate q_{NOx_N2} (mg N g VSS^{-1} h^{-1}) can be computed using the activated sludge MLVSS concentration (X_{VSS}):

$$q_{NOx\_N2} = 60 \cdot F_{NO3\_N2} / (V_{ML} \cdot X_{VSS}) \qquad \text{Eq. 2.4.53}$$

Test DEN.TIT Denitrification titrimetric test: assessing the denitrification kinetic rate

Activated sludge preparation

For conventional activated sludge samples from wastewater treatment plants treating urban wastewaters, a sample with X_{VSS} of around 2-4 g VSS L^{-1} will be suitable. The sample should be collected at the outlet or end of the pre-denitrification or post-denitrification tank, depending on the target treatment section.

1. Pour a known volume of the activated sludge sample (typically 1 L) into the reaction vessel and start the temperature control systems. Select the desired pH and temperature set point values.
2. Start the automatic titration system and leave the activated sludge under these conditions for a pre-incubation period (ideally 1 h). The pre-incubation phase will encourage the consumption of any residual nitrate or nitrite remaining from the plant.

Test execution

1. Start the data logging.
2. Add the nitrate stock solution (after adjusting its temperature to the target temperature of the test) to achieve a non-limiting nitrate concentration in the activated sludge (between 10 and 20 mg N L^{-1} are typically adequate values) and the S_B solution at a

concentration that is neither limiting nor inhibiting. Note that the amount of nitrate added ($M_{NOx,ini}$ in mg N) has to be known. To assess the appropriate amount of S_B, the stoichiometric relationship between the amount of S_B and nitrate consumed can be used, which can be calculated as follows:

$$Y_{NO3\_SB,Ax} = \frac{2.86}{1 - Y_{OHO,Ax}} \text{ (g COD g N}^{-1}\text{)} \quad \text{Eq. 2.4.38}$$

Note that the same type of test can be performed using nitrite instead of nitrate. In this case, the following relationship should be applied:

$$Y_{NO2\_SB,Ax} = \frac{1.71}{1 - Y_{OHO,Ax}} \text{ (g COD g N}^{-1}\text{)} \quad \text{Eq. 2.4.54}$$

The S_B addition can therefore be calibrated in order to guarantee that the S_B to nitrate (or nitrite) ratio is at least 3-4 times the stoichiometric value reported above. Note that Y_{HD} depends on the carbon source. Nevertheless, a value of 0.5 can be typically used for this rough estimation. This means that the S_B addition should be calibrated in order to ensure a S_B concentration in the activated sludge of 350 mg COD L^{-1} when using nitrate and 200 mg COD L^{-1} when using nitrite. Upon these additions, denitrifying bacteria will become active and their activity will usually tend to increase the pH. The automatic titration system will react to decrease the pH to maintain the pH set point through acid addition.

3. Record the volume of titration solution added over time (V_{tit} versus time). Check that the pH value remains within the interval pH set point ± 0.02. DO concentration should be below the detection limit. Continue the test until a clear bending point is observed in the cumulated titration solution plot. This bending point indicates that the nitrate is fully depleted and consequently denitrification has stopped. The collected data should be sufficient to reliably estimate the titration rate (Q_{tit}) from a linear regression of V_{tit} versus time with a satisfactory coefficient of determination (normally $R^2 > 0.98$).
4. Step 3 can be repeated by adding another dose of nitrate. The addition of S_B is no longer needed since a sufficient residual concentration is still present in the activated sludge to support a second denitrification phase.
5. End the test according to the instructions reported in Step 4 of the test operation procedure described for Test NIT.TIT.1.

Data analysis

A typical trend in the cumulated volume of titration solution added during the test is depicted in Figure 2.4.9.

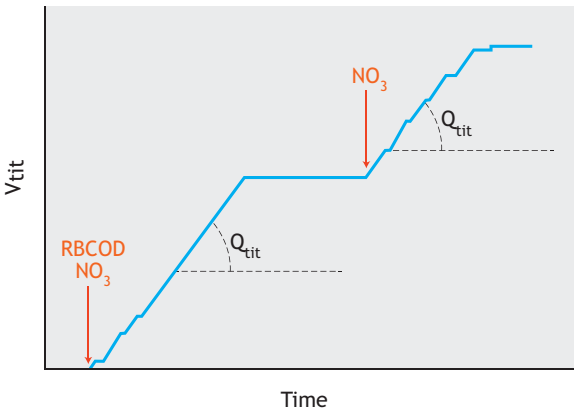

Figure 2.4.9 Example of a pH-static titration curve during a denitrification test to assess the maximum denitrification rate. Arrows indicate the addition of nitrate and S_B solutions. The relevant titration rate and is also shown in the graph.

From the data (V_{tit} versus time data), the titration rates (Q_{tit}) can be computed based on the slope of the titration curve (see Section 2.4.3).

The $Y_{NO3\_H+,Ax}$, in g N mol Protons^{-1}, can be assessed by considering the volume of titration solution added until the plateau of the titration curve is observed (V_T in Figure 2.4.9) and the mass of nitrate (or nitrite) added ($M_{NOx,ini}$):

$$Y_{NO3\_H+,Ax} = \frac{M_{NOx,ini}}{V_T \cdot N_T} \quad \text{Eq. 2.4.55}$$

Where, N_T is the titration solution normality. When more than one spike is performed (as in the case shown in Figure 2.4.9), the calculation can be repeated per each spike and the mean value can be taken as the estimate of $Y_{NO3\_H+,Ax}$.

The titration rate (Q_{tit} in mL min^{-1}) can be used to assess the denitrification rate ($F_{NO3\_H+,Ax}$ in mg N min^{-1}) by taking into account the concentration of the titration solution (N_T) and the $Y_{NO3\_H+,Ax}$ value:

$$F_{NO3\_H+,Ax} = Q_{tit} \cdot N_T \cdot Y_{NO3\_H+,Ax} \quad \text{Eq. 2.4.56}$$

Finally, the maximum specific denitrification rate of the sludge (q_{NOx_N2}, in mg N g VSS^{-1} h^{-1}), is computed by taking into account the MLVSS concentration of the sludge sample (in g VSS L^{-1}) and the suspension volume assessed at the end of the test (in L):

$$q_{NOx\_N2} = 60 \cdot F_{NO3\_H+,Ax} / (V_{ML} \cdot X_{VSS}) \quad \text{Eq. 2.4.57}$$

2.4.8 Anammox batch activity tests: preparation

As described in Section 2.4.1, the anammox process consumes ammonium and nitrite and converts them mainly into nitrogen gas as well as a minor fraction of nitrate. When the anammox process takes place in a batch reactor then, variations in the ammonium, nitrite and nitrate concentrations are expected and can be monitored to follow the time evolution of the process. Furthermore, when a gas-tight reaction vessel is used to perform the batch test, the release of dinitrogen gas will cause a pressure increase, which can also be monitored over time to measure the reaction kinetics. Therefore, two alternatives are available to track the evolution of the anammox process:

- Chemical tracking, by assessing the ammonium/nitrite/nitrate concentration evolution in time.
- Manometric tracking by assessing the overpressure caused by dinitrogen release in a gas-tight reactor.

Each one of these alternatives is discussed hereafter in the coming two tests. The first test (AMX.CHE) refers to a batch test performed by chemical tracking to assess the maximum activity of an anammox culture fed with synthetic autotrophic medium. In this test, the stoichiometric coefficients NO_2/NH_4 and NO_3/NH_4 ratios will be also assessed. In the second test (AMX.MAN), anammox biomass is suspended in a real wastewater and a manometric tracking procedure is applied to evaluate its treatability via the anammox process. In this example, the inhibition potential of a wastewater is evaluated by comparing the maximum rate obtained in the presence of wastewater with the maximum rate observed in the presence of a synthetic medium.

2.4.8.1 Apparatus

Each tracking methodology (chemical or manometric) has special apparatus requirements.

When chemical tracking is applied, refer to the following list of equipment:

1. An (airtight) batch reactor equipped with a mixing system and adequate sampling ports (as described in Section 2.4.3.1).
2. A nitrogen gas supply (recommended).
3. A calibrated pH electrode (if not included/incorporated in the batch reactor setup).
4. A 2-way pH controller via HCl and NaOH addition (alternatively a one-way control - generally for HCl addition - or manual pH control can be applied through the manual addition of HCl and NaOH).
5. A thermometer (recommended working temperature range of 0 to 40 °C).
6. A temperature control system (if not included in the batch reactor setup).
7. A DO meter with electrode (if not included/incorporated in the batch reactor setup) to verify anoxic conditions.
8. A stopwatch.

When the manometric tests are performed, refer to the following list of equipment:

1. The equipment given for the manometric system, described in Section 2.4.3.3.
2. A nitrogen gas supply (recommended).

2.4.8.2 Materials

For a complete list of required materials refer to Section 2.2.3.2 (with the exception of points 7 and 8).

2.4.8.3 Working solutions

- **Real wastewater**

For batch tests that require the use of a real wastewater, follow the instructions given in Section 2.2.3.3.

- **Synthetic medium**

If tests can be or are desired to be performed with synthetic wastewater, the synthetic influent media could contain a mixture of ammonium, nitrite and bicarbonate plus necessary (macro- and micro-) nutrients. Generally, they can be mixed all together in the same media or prepared separately if they need to be added in different phases or times. The usual compositions and concentrations are (based on van de Graaf *et al*., 1996): 1 g L^{-1} $(NH_4)_2SO_4$ (7.6 mM, 106 mg N L^{-1}), 0.25 g L^{-1} $NaNO_2$ (3.6 mM, 51 mg N L^{-1}), 0.6 g L^{-1} $NaNO_3$ (7.1 mM, 99 mg N L^{-1}), 1 g L^{-1} $NaHCO_3$ (11.9 mM), 0.025 g L^{-1} KH_2PO_4 (0.18 mM), 0.1 g L^{-1} $MgSO_4 \cdot 7H_2O$ (0.41 mM), 0.15 g L^{-1} $CaCl_2 \cdot 2H_2O$ (1.02 mM) and 1.25 mL L^{-1} trace elements solutions A and B (see below for trace elements A and B preparation). Similar nutrient solutions

can be used as long as they contain all the previously reported required nutrients. The trace element solutions should contain all the required micro-nutrients (iron, zinc, cobalt, manganese, copper, molybdate, nickel, selenium and boron) to ensure that cells are not limited by their absence and avoid obtaining incorrect results and in extreme cases the failure of the test. Thus, despite the fact that their concentrations may seem very low, one must make sure that all of the constituents are added to the solution in the required amounts. The following composition (amounts per litre of micro-nutrient solution) is recommended (based on van de Graaf *et al*., 1996): Trace elements solution A: 5 g EDTA, 9.14 g $FeSO_4 \cdot 7H_2O$, trace element solution B: 15 g EDTA, 0.43 g $ZnSO_4 \cdot 7H_2O$, 0.24 g $CoCl_2 \cdot 6H_2O$, 0.99 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.22 g $Na_2MoO_4 \cdot 2H_2O$, 0.19 g $NiCl_2 \cdot 6H_2O$, 0.21 g $NaSeO_4 \cdot 10H_2O$, and 0.014 g H_3BO_3.

- **Ammonium or nitrite solutions**

For the dosage of the anammox substrates, ammonium and nitrite stock solutions can be prepared (e.g. 1-10 g N L^{-1}) using either $(NH_4)_2SO_4$ or NH_4Cl salts for ammonium and $NaNO_2$ or KNO_2 salts for nitrite. Generally, they can be mixed together in the same stock solution or prepared separately if they need to be added in different phases or at different times. When an ammonium/nitrite solution is prepared the molar ratio between the two substrates is usually set to one in order to ensure an excess of ammonium throughout the test. Bicarbonate can also be added to the batch test to avoid inorganic carbon (IC) limitation: in order to ensure IC in excess during the test, add bicarbonate to the ammonium solution up to a molar ratio equal to 0.7 mol-IC mol-NH_4^{-1} (i.e. ten times the stoichiometric requirements of 0.066 mol-IC mol-NH_4). The latter should be considered in case the wastewater tested has limiting concentrations of inorganic carbon and/or if the oxygen is removed via sparging CO_2-free gases (e.g. N_2), which will lead to CO_2 stripping.

- **Nitrate solution**

In order to ensure the correct redox conditions, nitrate can be dosed prior to the execution of the batch test using a nitrate stock solution that can be prepared (e.g. 1-10 g N L^{-1}) using either $NaNO_3$ or KNO_3 salts. This is especially needed when consecutive batch tests need to be performed using the same anammox sludge (e.g. to evaluate the long-term effect of exposure to a particular compound) in order to avoid sulphate reduction to sulphide (H_2S), which may be toxic to anammox bacteria.

- **Washing media**

If the sludge sample must be 'washed' to remove any undesirable compounds (which may even be inhibitory or toxic), a washing solution is needed. The synthetic medium described above can be used as a washing medium by simply removing the nitrite salts from the recipe. The washing process can be repeated twice or three times. Thereafter, the following preparation steps can be performed. In special cases, e.g. when sludge from a full-scale plant is used, the plant effluent may be used for washing purposes (assuming that its composition allows this).

- **Acid and base solutions**

These should be respectively 100-250 mL of 0.2 M HCl and 100-250 mL 0.2 M NaOH solutions for automatic or manual pH control, and 10-50 mL of 1 M HCl and 10-50 mL 1 M NaOH solutions for initial pH adjustment if the desired operational pH is very different from the pKa value of the buffering agent.

Finally, the required working and stock solutions to carry out the determination of the analytical parameters of interest must also be prepared in accordance with Standard Methods (APHA *et al.,* 2012) and the corresponding protocols.

It is recommended to take samples of the media and activated sludge prior to the execution of the experiment to confirm/check the initial (desired) concentrations of parameter(s) of interest (e.g. ammonium, nitrite, nitrate, X_{TSS} and X_{VSS}).

2.4.8.4 Material preparation

For general instructions on how to organize the material preparation, refer to Section 2.2.3.4 and to Table 2.2.2. If required, test-specific requirements will be listed within each test protocol.

2.4.9 Anammox batch activity tests: execution

Test AMX.CHE Anammox chemical test: assessing the maximum anammox kinetic rate and the stoichiometric coefficient NO_2/NH_4 and NO_3/NH_4 ratios

Activated sludge preparation

For a suitable sampling point for the activated sludge, refer to Section 2.4.3.4.

1. For conventional activated sludge samples from municipal wastewater treatment plants, a sample

containing a X_{VSS} of around 2-10 g L^{-1} will be suitable. The X_{VSS} can be adjusted as suggested in Section 2.4.3.5. For anammox granular sludge, instead of using the activated sludge, granular biomass can be easily separated from the supernatant by settling (in a cylinder or in an Imhoff cone) and re-suspended in a washing solution or in the effluent of the plant. Preliminary tests can be conducted to evaluate the density of the settled granular sludge (g VSS L^{-1}) to pour a defined amount of anammox granular sludge (g VSS) into the reaction vessel.

2. Pour a defined volume of the activated sludge sample (typically 1 to 3 L) or a known amount of anammox granular sludge (re-suspended in the medium to be tested) into the reaction vessel and start mixing. Also, start the temperature and pH control systems to maintain both parameters around the desired set point values.
3. Sparge N_2 (or N_2/CO_2 gas mixture, see Section 2.4.3.5) into the headspace for approximately 10 min to ensure an oxygen-free environment. Install a gas outlet to limit the overpressure. Gas sparging can continue until the end of the test. If this is not feasible, then an airtight reactor with a device able to prevent oxygen intrusion can be used (e.g. using a unidirectional check valve or a water lock) (see Section 2.2.2.1 for further details).
4. Dose nitrate up to a final concentration in the activated sludge of 50-100 mg N L^{-1} in order to ensure an adequate redox potential and avoid sulphate reduction.
5. Wait for approximately 30 min to ensure stable initial conditions. This pre-incubation phase will normally allow the removal of any residual nitrite present.

Test execution

1. Verify that the temperature, pH and DO target values are within the desired intervals. Otherwise adjust them and wait until they stabilize.
2. Add the ammonium/nitrite stock solution (previously adjusted to the target temperature) to achieve a neither limiting nor inhibiting nitrite concentration in the activated sludge: 50 to 75 mg N L^{-1} is usually adequate for anammox biomass cultivated under non-strict nitrite limiting conditions (see Section 2.4.3.6 for further explanations). If any residual nitrite is present in the activated sludge sample, the nitrite addition should be reduced accordingly. The ammonium starting concentration is less critical considering the concentration ranges typical of anammox systems due to the lower inhibiting effect on anammox bacteria: 50 to 200 mg N L^{-1} is usually considered as adequate.
3. Start the stopwatch just after the addition of the ammonium/nitrite to track the sampling times.
4. Collect the activated sludge samples at regular time intervals. For instance, collect the samples every 20 to 30 min throughout the duration of the test (which usually lasts around 3 to 4 h).
5. Conclude the test when the nitrite is fully depleted. Use nitrite strip tests to quickly estimate the nitrite concentrations.
6. Take a sample to determine the final MLVSS concentration.

Note that the nitrite concentrations are unstable, and therefore they need to be quickly determined after sampling.

Data analysis

A typical output of this test is shown in Figure 2.4.10.

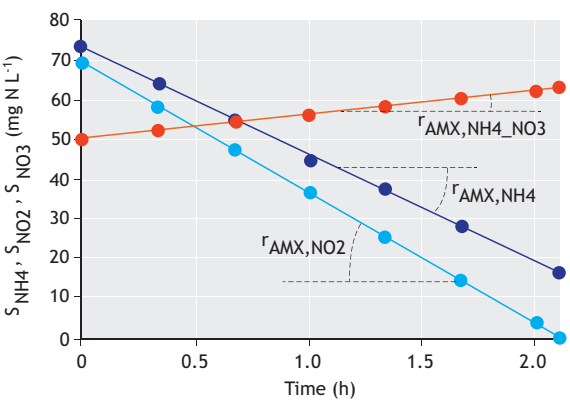

Figure 2.4.10 Typical N profiles in a Test AMX.CHE: ammonium (●), nitrite (●) and nitrate (●) concentrations are displayed on the y-axis. The relevant rates of interest are also indicated (ammonium removal rate $r_{AMX,NH4}$, nitrite removal rate $r_{AMX,NO2}$, and nitrate production rate $r_{AMX,NH4\_NO3}$).

The linear regression over these data (see Section 2.4.5) can be used to determine the ammonium ($r_{AMX,NH4}$) and nitrite removal rates ($r_{AMX,NO2}$) as well as the nitrate production ($r_{AMX,NH4\_NO3}$) rate (expressed as mg N L^{-1} h^{-1}). The anammox rate is usually expressed as the dinitrogen gas produced, which is equivalent to the nitrogen removed from the wastewater. The maximum specific anammox rate ($q_{AMX,N2}$ as mg N_2-N g VSS^{-1} h^{-1}) can therefore be computed as follows:

$$q_{AMX,N2} = \frac{r_{AMX,NH4} + r_{AMX,NO2} - r_{AMX,NH4\_NO3}}{X_{VSS}} \quad \text{Eq. 2.4.58}$$

In the previous expression, when anammox granular sludge is used to perform the test, then the biomass concentration at the denominator (the term X_{VSS}) will correspond to the g VSS added to the reactor vessel during the test preparation divided by the mixed liquor volume.

The stoichiometric coefficient ratios of interest, such as $Y_{NH4\_NO2,AMX}$ and $Y_{NH4\_NO3,AMX}$, can be easily calculated from the relative removal/production rates using the following expressions:

$$Y_{NH4\_NO2,AMX} = \frac{r_{AMX,NO2}}{r_{AMX,NH4}} \quad \text{Eq. 2.4.59}$$

$$Y_{NH4\_NO3,AMX} = \frac{r_{AMX,NH4\_NO3}}{r_{AMX,NH4}} \quad \text{Eq. 2.4.60}$$

Test AMX.MAN Anammox manometric test: assessing the maximum anammox kinetics

Activated sludge preparation

1. Follow step 1 of the activated sludge preparation protocol described in Test AMX.CHE above.
2. Select an appropriate activated sludge volume to be poured into the reaction vessel (V_{ML}). For anammox granular sludge, see the instructions described in Test AMX.CHE. In this test, the overpressure depends on the ratio between the amount of ammonium to be oxidized (which is based on the stoichiometry of the reaction; 1 mol of NH_4 consumption will lead to 1 mol of N_2 production) (see Section 2.4.1) and the headspace volume (V_{HS} in L). Hence, the correct selection of this ratio is crucial to avoid the generation of an extreme overpressure (either too low or too high). The maximum overpressure will be achieved at the end of the test, i.e. when nitrite (usually the limiting substrate) has been fully converted. Considering a $Y_{NH4\_NO2,AMX}$ stoichiometric ratio of 1.32 mol-NO_2 mol-NH_4^{-1}, the maximum overpressure (P_{max}) can be estimated as follows:

$$P_{max} - P_{atm} = \frac{P_{atm}}{V_{HS}} \cdot \frac{M_{NO2\_N2}}{14} \cdot 22.4 \cdot \frac{(273 + T_C)}{273} \quad \text{Eq. 2.4.61}$$

Where, M_{NO2_N2} (in mg N) is the mass of nitrite that is converted during the course of the test, dependent on the initial nitrite concentration ($S_{NO2,ini}$, in mg N L^{-1}) and activated sludge volume (V_{ML} in L):

$$M_{NO2\_N2} = S_{NO2,ini} \cdot V_{ML} \quad \text{Eq. 2.4.62}$$

By substituting this equation into the previous one and rearranging, the following relationship is obtained:

$$\frac{V_{ML}}{V_{HS}} = \frac{P_{max} - P_{atm}}{P_{atm}} \cdot \frac{14 \cdot 1.32}{22.4} \cdot \frac{273}{(273 + T_C)} \cdot \frac{1{,}000}{S_{NO2,ini}} \quad \text{Eq. 2.4.63}$$

The optimal P_{max} value usually lies around 0.2 atm, which means that, under typical test conditions (T_C ~30 °C, $S_{NO2,ini}$ ~50 mg N L^{-1}), a V_{ML} to V_{HS} ratio of around 3 is obtained. Therefore, for a total reactor volume of 1 L ($V_{TOT} = V_{ML} + V_{HS}$), an adequate activated sludge volume will be 0.75 L.

3. Pour the previously estimated activated sludge volume into the reaction vessel. Place the reaction vessel on the shaker (see Section 2.4.3.1 for mixing requirements). Flush the reactor headspace with N_2 (or a N_2/CO_2 gas mixture, see Section 2.4.3.3) and seal the gas bottle tightly. Place the reactor into the thermostatic chamber, apply a gentle mixing and wait for 30 min until the temperature stabilizes.

Test execution

1. Select the volume of nitrite solution to be dosed at the beginning of the batch test to achieve a neither limiting nor inhibiting nitrite concentration in the activated sludge: typically, 50 to 75 mg N L^{-1} are adequate concentrations for anammox biomass cultivated under non-strict nitrite limiting conditions (see Section 2.4.3.4 for further details). Usually, the initial ammonium concentration is similar to the nitrite concentration. Higher initial ammonium concentrations can be used, as long as they remain below the reported inhibitory level for anammox bacteria (< 1 g N L^{-1}). If there is residual nitrite present in the activated sludge sample, the nitrite addition should be reduced accordingly. Using a syringe, inject the ammonium/nitrite stock solution through the rubber septum.
2. Start the manometric data collection. Collect the data every 30 to 60 min until there is a point where the overpressure curve bends, which will correspond to the exhaustion of nitrite.
3. End of the test. Check the final pH and take a final sample to determine the MLVSS concentration.

Data analysis

Using the ideal gas law, the pressure data can be converted into the gas moles (N_2 in this case) emitted to the headspace:

$$n(t) = \frac{P(t) \cdot V_{HS}}{R \cdot T_K} \qquad \text{Eq. 2.4.64}$$

Where, n(t) is the number of N_2 moles present in the headspace volume (V_{HS}) at time t, P(t) is the pressure in the headspace at time t, R is the ideal gas constant and T_K the temperature of execution expressed in Kelvin.

Figure 2.4.11 shows a typical profile of a manometric anammox test executed with a manometer equipped with a data logger after the conversion of the recorded pressure data into moles of N_2 gas produced. Disregard the data collected in the first 10-15 min of the test since various phenomena may overlap affecting the headspace overpressure, such as: (*i*) residual oxygen intrusion from the headspace, (*ii*) water-vapour pressure equilibrium; (*iii*) initial N_2 accumulation in the liquid phase, and (*iv*) microbiological lag phases.

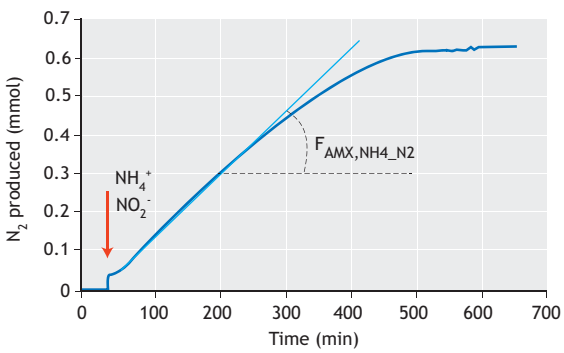

Figure 2.4.11 Typical N_2 gas profiles obtained in a Test AMX.MAN performed with a manometer equipped with a data logger. The maximum N_2 production rate $F_{AMX,NH4\_N2}$ is depicted in the figure.

The cumulative N_2 production curve can be used to compute the N_2 production rate ($F_{AMX,NH4\_N2}$, N_2-mol min^{-1}) using linear regression (see Section 2.4.5). Moreover, data can be further expressed in mg N_2-N min^{-1} using the equivalent molecular weight of dinitrogen gas (28 g N N_2-mol^{-1}). The specific anammox rate $q_{AMX,N2}$ (mg N g VSS^{-1} h^{-1}) can be computed using the MLVSS concentration:

$$q_{AMX,N2} = 60 \cdot F_{AMX,NH4\_N2} / (V_{ML} \cdot X_{VSS}) \qquad \text{Eq. 2.4.65}$$

2.4.10 Examples

2.4.10.1 Nitrification batch activity test

Description

To illustrate the execution of an aerobic batch activity test for nitrification, data from a test performed at 20ºC with a full-scale activated sludge sample is presented in this section. The Test NIT.CHE.1 was carried out to determine the maximum specific biomass ammonium oxidation rate. The batch activity test was performed using a 2.5 L reactor. All the equipment, apparatus and materials were prepared as described in Section 2.4.4.1. The pH and DO sensors were calibrated less than 24 h before the test execution. The batch test lasted 3 h. Including the time needed for the test preparation and the cleaning phase afterwards, the operations lasted approximately 5 h. Prior to the batch test, 2.0 L of fresh activated sludge collected at the end of the aerobic phase of a full-scale plant (according to Section 2.4.3.4) was transferred to the reactor and acclimatized for 1 h at 20 ˚C under slow mixing (100 rpm) following the recommendations described in Section 2.4.3.5. The pH and DO control were started up at pH and DO set points of 7.5 and 6 mg O_2 L^{-1}, respectively. Thereafter, 15 min before the start of the test, mixing was increased to 200 rpm and samples were collected for the determination of the parameters of interest (e.g. ammonium, nitrite, nitrate and MLVSS concentrations). Before the addition of the synthetic medium, its temperature was adjusted in a water bath to the target temperature of the batch test (20 ˚C). The first sample was collected 5 minutes before the addition of the synthetic medium to determine the initial conditions. The test started with the addition of the synthetic media (minute zero): 50 mL containing 1 g NH_4-N L^{-1} as well as other macro- and micro-nutrients as described in Section 2.4.3.6. Samples were collected every 30 min over a period of 3 h. The duration of the batch test was chosen in order to allow a complete oxidization of the ammonium present. Immediately after collection, all the samples were prepared and preserved as described in Section 2.2.3.4. All the collected samples were analysed as described in Section 2.4.3.7. Table 2.4.5 shows the experimental implementation plan and the results of the execution of the test.

Data analysis

Following on from the results from the experiment shown in Table 2.4.5, Figure 2.4.12 shows the results from the test and also an estimation of the maximum volumetric kinetic rates (by applying linear regression).

Table 2.4.5 Results from the nitrification batch activity test.

Nitrification batch activity test **Code: NIT.CHE.1**

Date:	Monday 05.10.2015 10:00 h
Description:	Nitrification test at 20 °C with real activated sludge
Test No.:	1
Duration	3 h (180 min)
Substrate:	Synthetic: ammonium and nitrite (1,000 mg L^{-1}) + minerals
Sampling point:	Middle mixed liquor height in the SBR
Samples No.:	NIT.CHE 1-8
Total sample volume:	80 mL (5 mL normal sample, 20 mL for MLVSS)
Reactor volume:	2.5 L

Experimental procedure in short:	**Time (h:min)**
1. Confirm availability of sampling material and required equipment.	08:00
2. Confirm calibration and functionality of systems, meters and sensors.	08:10
3. Transfer 2.0 L of sludge to batch reactor.	08:30
4. Start aerobic conditions with gentle mixing and air sparging at T and pH set points.	08:40
5. 15 min before starting, take sample for initial conditions (sample NIT.CHE 1).	09:40
7. Start batch test: add 0.05 L of synthetic influent.	09:55
8. Take first sample for the determination of the initial conditions (minute zero)	10:00
9. Minute 30, continue sampling program according to schedule	10:30
10. Minute 180, stop aeration and mixing.	13:00
11. Organize the samples and clean the system.	13:10
12. Verify that all systems are swtiched off.	13:20

Sampling schedule

Time (min)	-15	-5	0	30	60	90	120	150	180
Time (h)	-0.25	-0.08	0.00	0.50	1.00	1.50	2.00	2.50	3.00
Sample No.	1		2	3	4	5	6	7	8
Parameter	AEROBIC PHASE								
NH_4-N (mg N L^{-1})	6.8		31	27.3	20.6	17.2	12.4	4.9	0.4
NO_2-N (mg N L^{-1})	0[1]		0	0.4	0.9	0.6	0.3	0.4	0.1
NO_3-N (mg N L^{-1})	0.1		0.1	5.1	9.8	14.2	19.4	24	29.6
MLSS and MLVSS (mg L^{-1})	See table								See table

[1] Average value of the concentration present in the synthetic substrate and in the liquid phase of the sludge sample prior the start of the test

MLSS & MLVSS measurements

Sampling point	Cup No.	W1	W2	W3	W2-W1	W2-W3	MLSS	MLVSS	Ratio
Start test[2]	1	0.09630	0.16530	0.10210	0.06900	0.06320	3,450	3,160	0.92
	2	0.09580	0.16380	0.10190	0.06800	0.06190	3,400	3,095	0.91
	3	0.09640	0.16440	0.10230	0.06800	0.06210	3,400	3,105	0.91
						Average	3,417	3,120	0.91
End test	4	0.09540	0.16490	0.10200	0.06950	0.06290	3,475	3,145	0.91
	5	0.09610	0.16410	0.10180	0.06800	0.06230	3,400	3,115	0.92
	6	0.09570	0.16400	0.10220	0.06830	0.06180	3,415	3,090	0.90
						Average	3,430	3,117	0.91

[2] Concentrations corrected considering the dilution due to the synthetic medium addition.

Biomass composition

Sampling point	Start test	End test
MLSS (mg L^{-1})	3,417	3,430
MLVSS (mg L^{-1})	3,120	3,117
Ratio	0.91	0.91
Ash (mg L^{-1})	297	313

The maximum volumetric rates for ammonium removal and nitrate production are 10.3 and 9.7 mg N L^{-1} h^{-1}, respectively. Taking into account the average MLVSS concentration of 3.1 g L^{-1} between the sludge samples collected at the beginning and end of the test, a biomass-specific ammonium oxidation rate of 3.3 mg N g VSS^{-1} h^{-1} can be estimated, which corresponds to about 80 mg N g VSS^{-1} d^{-1}. It is important to note that the sum of soluble inorganic nitrogen compounds at the beginning and end of the batch test are comparable (31.1 vs 30.1 mg N L^{-1}), indicating that nitrification was the dominant process during the batch test.

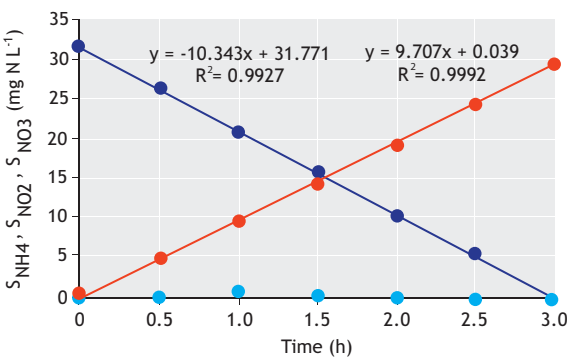

Figure 2.4.12 Graphic representation of the ammonium (●), nitrite (●) and nitrate concentrations (●) obtained in the example of the batch activity nitrification test (Test NIT.CHE.1). The test was performed with a full-scale activated sludge at 20 °C and pH 7.5 using a synthetic medium as influent.

The two trend lines show the ammonium conversion and nitrate production rates for further estimation of the corresponding maximum specific biomass kinetic rates. A decrease in the sum of ammonium, nitrite and nitrate concentrations would suggest the simultaneous occurrence of nitrogen removal processes such as denitrification or anammox, which can take place in the presence of anoxic conditions. The comparable ammonium oxidation and nitrate production rates indicate that, in the tested sludge, the activities of AOO and NOO are well-balanced and allowed to achieve full nitrification. These observations were also supported by the absence of nitrite. The measured specific ammonium oxidation rate is comparable to the kinetic rates reported in Table 2.4.1, indicating a high enrichment of nitrifying bacteria in the activated sludge sample tested.

2.4.10.2 Denitrification batch activity test

Description

In this section, a test to assess the maximum denitrification rate and the anoxic growth yield in the presence of acetate as the carbon source is described.

An airtight reactor equipped with a mechanical mixing system and automatic pH and temperature control systems was used. N_2 gas bubbling was also provided.

The day before testing, the pH and DO probes were calibrated and all the required materials were prepared as suggested in Section 2.2.3.2 (with the exception of points 7 and 8). A 10 g N L^{-1} nitrate solution was prepared with $NaNO_3$ and an acetate stock solution using sodium acetate at a concentration of 10 g COD L^{-1} (taking into account that 1 g CH_3COONa corresponds to 0.78 g COD). 1 M HCL and 1 M NaOH solutions were prepared for automatic pH correction. Moreover, 2 L of sludge were used. A preliminary working plan was defined as displayed in Table 2.4.6.

Table 2.4.6 Results from the denitrification batch activity test.

Denitrification batch activity test — **Code: DEN.CHE.1**

Date:	Wednesday 02.09.2015 9:00 h	**Experimental procedure in short:**	**Time (h:min)**
Description:	Tests at 20 °C, pH 7, artificial substrate & enriched lab culture	1. Day before instrumentation check, probes calibration preparation of the working plan, stock solutions, containes for sample collections, and all other materials.	
Test No.:	1		
Duration	3.5 h (210 min)	2. Sludge sampled from SBR and transfered to reaction vessel.	09:00
Substrate:	Synthetic: Acetate (200 mgCOD/L) + nitrate (20 mgN/L)	3. Activation of N_2 sparging.	09:05
Sampling point:	Middle mixed liquor height in the SBR	4. Nitrate addition, fist sample taken.	09:10
Samples No.:	DEN.CHE.1 (1-18)	5. Other 5 samples taken at 20 min intervals.	
Total sample volume:	240 mL (10 mL for MLVSS, and 10 mL each sample)	6. Acetate addition and sampling.	10:30
		7. Other 13 samples taken at 5/10 min intervals.	
Reactor volume:	2.5 L	8. Test stopped.	11:35
		9. Sample for MLVSS measurement taken, total volume assessment.	11:40
		10. Verify that all samples are correctly stored and switch off system.	11:50

Sampling schedule

Time (min)	0	20	40	60	80	85	90	95	100	105	110	120
Time (h)	0.00	0.33	0.67	1.00	1.33	1.42	1.50	1.58	1.67	1.75	1.83	2.00
Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Parameter	ANOXIC PHASE											
NO_3-N (mg N L^{-1})	23.0	23.2	22.0	21.8	20.2	18.2	15.8	14.0	12.3	11.2	10.2	7.3
NO_2-N (mg N L^{-1})	0.1	0.0	0.2	0.0	0.0	1.0	2.0	2.5	2.8	3.0	3.0	2.7
$COD_{soluble}$ (mg COD L^{-1})					418.0	407.3	397.3	385.8	378.0	366.5	353.7	336.5

Sampling schedule (continued)

Time (min)	130	140	150	160	180	185
Time (hrs)	2.17	2.33	2.50	2.67	3.00	3.08
Sample No.	13	14	15	16	17	18
Parameter	ANOXIC PHASE					
NO_3-N (mg N L^{-1})	4.9	1.8	0.3	0.0	0.0	
NO_2-N (mg N L^{-1})	2.0	2.1	1.5	1.0	0.0	
$COD_{soluble}$ (mg COD L^{-1})	315.0	303.6	285.7	275.0	271.4	
MLSS (g L^{-1})						2.51

Total biomass (gVSS)	4.58
$r_{D,end}$ (mg N L^{-1} min^{-1})	0.035
$r_{D,exog}$ (mg N L^{-1} min^{-1})	0.278
r_{COD} (mg COD L^{-1} min^{-1})	2.050
Anoxic growth yield	0.660
Denitrification rate (mg N g $MLVSS^{-1}$ h^{-1})	6.600

The initial nitrate concentration was set to 20 mg N-NO_3 L^{-1} (corresponding to the addition of 4 mL of nitrate stock solution). The initial COD concentration was set to 200 mg L^{-1} (an addition of 40 mL of acetate stock solution). According to the instructions reported in Section 2.4.7, up to 18 samples were planned to be collected. All corresponding materials and consumables (e.g. plastic cups) were prepared and labelled to avoid identification errors during the sample collection. On the day of the test, the activated sludge was sampled from a bench scale SBR at the end of its denitrification phase. Two litres of activated sludge were transferred to the reaction vessel and the temperature set point was set at 20 °C, similar to the operating temperature of the bench-scale SBR. Simultaneously, the pH-control system was started up with a set point value of 7.4. The initial pH was 7.7. The N_2 gas sparging system was turned on to remove any residual DO and ensure anoxic conditions after the nitrate addition. After 5 min, the S_{O2} concentration in the bulk liquid was below the probe detection limit and the N_2 sparging was turned off. The predefined volume of the nitrate stock solution was spiked through the sampling port. After approximately 1 min, the stopwatch was turned on and the first sample was taken (minute zero). Four more samples were taken at 20 min intervals to assess the endogenous denitrification rate. Thereafter, the COD stock solution was added and, 30 seconds afterwards, a sample was taken. Later on, 6 samples were taken at 5 min intervals and afterwards samples were taken every 10 min. The sampling campaign continued while the nitrate and nitrite profiles were continuously followed using NO_3 and NO_2 detection paper strips until the nitrate and nitrite were fully consumed. These samples were used to assess the nitrate, nitrite, and soluble COD concentration. The test finished after 180 min. One final sample was taken to assess the MLVSS concentration. Eventually, all the systems were stopped and the reactor was opened in order to measure the final volume of activated sludge. All the samples were collected and preserved as described in Section 2.2.3.4. All the collected samples were analysed as described in Section 2.4.3.7.

Data analysis

Relevant implementation data and results of the analytical determinations are reported in Table 2.4.6. In Figure 2.4.13, the COD and $S_{NO3,Eq}$ (computed as $S_{NO3,Eq} = S_{NO3} + 0.6 \cdot S_{NO2}$) trends are plotted.

As shown in Figure 2.4.13, the endogenous and exogenous denitrification rates were estimated by linear regression ($r_{NOx\_N2,exo}$ and $r_{NOx\_N2,endo}$ in mg N L^{-1} min^{-1}), as well as the exogenous COD consumption rate (r_{COD}, in mg COD L^{-1} min^{-1}). Note that only data obtained in the non-limiting denitrification phase were used for the determination of the kinetic rates. For example, only those data that led to the highest linear fitting, based on the highest R^2 value, for both N-NO_{eq} and soluble COD.

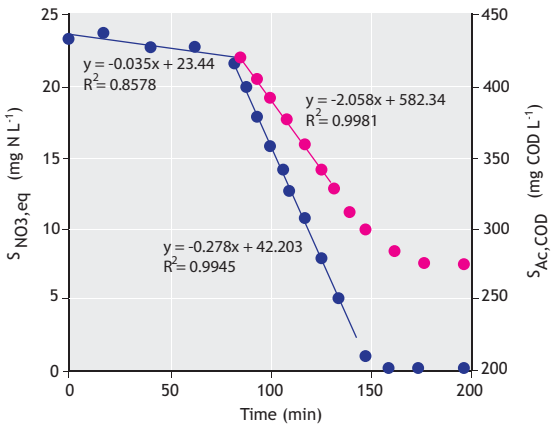

Figure 2.4.13 Graphic representation of (●) the N-NOeq and (●) soluble COD concentrations obtained in the Test DEN.CHE.1 (as presented in Table 2.4.6). The test was performed using an activated sludge sample from a SBR operated at 20 °C and pH 7.5 using acetate as the carbon source. The slopes of the trend lines were used to quantify the endogenous, exogenous denitrification and organic carbon uptake rates.

Thus, the maximum rates can be determined as:

- Maximum specific denitrification rate:

$$q_{Nox\_N2} = 60 \cdot \frac{r_{NOx\_N2,exo} - r_{NOx\_N2,endo}}{X_{VSS}}$$

$$= 60 \cdot \frac{0.28 - 0.035}{2.51} = 5.8 \text{ mg N g VSS}^{-1}\text{h}^{-1} \qquad \text{Eq. 2.4.66}$$

- Anoxic growth yield on acetate:

$$Y_{OHO,Ax} = 1 - 2.86 \cdot \frac{r_{NOx\_N2,exo} - r_{NOx\_N2,endo}}{r_{COD}}$$

$$= 1 - 2.86 \cdot \frac{0.28 - 0.035}{2.05}$$

$$= 0.66 \text{ g COD}_{biomass} \text{ g COD}_{acetate}^{-1} \qquad \text{Eq. 2.4.67}$$

Both obtained values are in the range of data reported in Table 2.4.2, supporting the reliability of the results.

2.4.10.3 Anammox batch activity test

Description

To illustrate the execution of an anammox batch activity test, data from a test performed at 30 °C with a full-scale anammox biomass sample is presented in this section. The Test AMX.CHE was carried out to determine the maximum specific biomass anammox rate (k_{AMX}, mg N g $MLVSS^{-1}$ d^{-1}) as well as the stoichiometric parameters of interest for nitrite consumption and nitrate production with respect to ammonium consumption (NO_2/NH_4 and NO_3/NH_4 ratios described in Section 2.4.3.8). The batch activity test was performed using a 2.5 L reactor. All the equipment, apparatus and materials were prepared as described in Section 2.4.3. The pH sensor was calibrated less than 24 h before the test execution. The batch test lasted for 3 h. Including the time needed for the test preparation and the cleaning phase afterwards, the operations lasted for approximately 5 h. Prior to the batch test, 1 L of fresh anammox sludge collected at a full-scale 1-stage PN/anammox plant (according to Section 2.4.3.4) was transferred to the reactor and acclimatized for 1 h at 30 °C under slow mixing (100 rpm) following the recommendations described in Section 2.4.9. To ensure an oxygen-free environment, a N_2/CO_2 gas mixture (see Section 2.4.3.5) was sparged into the bulk liquid in the first 10 min and into the reaction headspace throughout the batch test. A gas outlet was provided to limit any overpressure and prevent oxygen back-diffusion (e.g. using a water lock). The pH control was started with a pH set point of 7.5. Thereafter, 15 min before the start of the test, mixing was increased to 200 rpm and samples were collected to characterize the anammox sludge used in the test (e.g. ammonium, nitrite, nitrate and MLVSS concentrations). The first sample was collected 5 min before the addition of a synthetic medium in order to determine the initial conditions. The test started with the addition of 70 mL of the synthetic medium containing 1 g NH_4-N L^{-1} and 1 g NO_2-N L^{-1} as well as other macro- and micro-nutrients as described in Section 2.4.9 (minute zero). In the example, nitrate was not dosed at the beginning of the test since it was already present in the anammox sludge sample. Before its addition, the temperature of the synthetic medium was adjusted in a water bath to the target temperature of the test (30 °C). Samples were collected every 30 min for a period of 3 h.

The maximum activity should be evaluated under non-limiting conditions. Considering the half-saturation constant for nitrite of 0.035 mg N L^{-1} reported in literature (in most cases the limiting substrate) (Lotti *et al.,* 2014), non-limiting nitrite concentrations are in the order of 1-2 mg N L^{-1} for flocculent or suspended anammox biomass. For biomass types characterized by higher mass transfer limitations such as biofilms attached to inert carriers and granular sludge, non-limiting conditions can be ensured at a nitrite concentration in the order of 5-10 mg N L^{-1}, depending on the density of the biofilm and on the specific anammox activity of the biofilms. When detailed information on the mass transfer characteristics of the biomass used during the batch test is not well known, one should consider for data analysis only those concentrations that can be satisfactory interpolated by linear regression ($R^2 > 0.95$). Immediately after collection, all the samples were prepared and preserved as described in Section 2.2.3.4. All the collected samples were analysed as described in Section 2.4.3.7. Table 2.4.7 shows the experimental implementation plan of the execution of the test.

Data analysis

Following the results from the experiment shown in Table 2.4.7, Figure 2.4.14 shows the results from the test displayed in the implementation plan and also an estimation of the maximum volumetric kinetic rates.

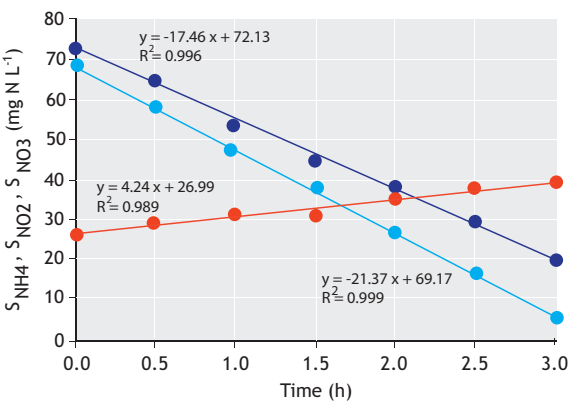

Figure 2.4.14 Typical output of an anammox batch activity test (type AMX.CHE) - ammonium (●), nitrite (●) and nitrate (●) concentrations observed during the test, resulting in depicted nitrogen conversion rates, namely: ammonium removal rate ($r_{AMX,NH4}$), nitrite removal rate ($r_{AMX,NO2}$), and nitrate production rate ($r_{AMX,NH4\_NO3}$). Note: data used in the figure were obtained from another test than presented in Table 2.4.7.

The maximum volumetric ammonium and nitrite removal, and the nitrate production rates are 17.5, 21.4 and 4.2 mg N L^{-1} h^{-1}, respectively. Taking into account the average MLVSS concentration of 2.2 g L^{-1} between the sludge samples collected at the beginning and end of

the test, a specific biomass ammonium oxidation rate ($q_{AMX,NH4\_N2}$) of 7.9 mg N g VSS^{-1} h^{-1} can be estimated. This rate corresponds to 189 mg N g VSS^{-1} d^{-1}. Using the same approach, the specific biomass nitrite reduction ($q_{AMX,NO2\_N2}$) and nitrate production ($q_{AMX,NH4\_NO3}$) rates can be calculated resulting in the maximum specific rates of 9.7 and 1.9 mg N g VSS^{-1} h^{-1}, respectively, which corresponds to 232 and 46 mg N g VSS^{-1} d^{-1}, respectively. Finally, the maximum volumetric nitrogen removal rate can be calculated as the sum of the maximum volumetric ammonium and nitrite removal rate minus the maximum volumetric nitrate production, obtaining a value of 830 mg N L^{-1} d^{-1}. Similarly, the maximum specific biomass nitrogen removal rate can be computed, which results in a specific biomass kinetic rate ($q_{AMX,N2}$ or specific anammox activity - SAA) of 375 mg N g VSS^{-1} d^{-1}. Thus, the $Y_{NH4\_NO2,AMX}$ and $Y_{NH4\_NO3,AMX}$ ratios can be calculated by dividing the corresponding volumetric (or specific biomass) kinetic rates.

Table 2.4.7 Results from the anammox batch activity test.

Anammox batch activity test — **Code: AMX.CHE**

Date:	Tuesday 13.10.2015 10:00 h
Description:	Anammox test at 30 °C with real activated sludge
Test No.:	1
Duration	3 h (180 min)
Substrate:	Synthetic: ammonium and nitrite (1,000 mg L^{-1} each) + minerals
Sampling point:	Middle mixed liquor height in the SBR
Samples No.:	AMX.CHE 1-8
Total sample volume:	80 mL
	(5 mL normal sample, 20 mL for MLVSS)
Reactor volume:	2.5 L

Experimental procedure in short:	**Time (h:min)**
1. Confirm availability of sampling material and required equipment.	08:00
2. Confirm calibration and functionality of systems, meters and sensors.	08:10
3. Transfer 1.0 L of sludge to batch reactor.	08:30
4. Start aerobic conditions with gentle mixing and air sparging at T and pH set points.	08:40
5. 15 min before starting, take a sample for initial conditions (AMX.CHE 1).	09:40
7. Start batch test: add 0.07 L of synthetic influent.	09:55
8. Take first sample for the determination of the initial conditions (minute zero)	10:00
9. Minute 30, continue sampling program according to schedule	10:30
10. Minute 180, stop mixing.	13:00
11. Organize the samples and clean the system.	13:10
12. Verify that all systems are swtiched off.	13:20

Sampling schedule

Time (min)	-15	-5	0	30	60	90	120	150	180
Time (h)	-0.25	-0.08	0.00	0.50	1.00	1.50	2.00	2.50	3.00
Sample No.	1		2	3	4	5	6	7	8
Parameter	ANAEROBIC PHASE								
NH_4-N (mg N L^{-1})	8.2		73.1	63.9	52.7	44.8	38.1	29.5	19.4
NO_2-N (mg N L^{-1})	0[1]		69.1	58.3	47.6	38.1	25.8	16.2	4.7
NO_3-N (mg N L^{-1})	27.5		27.2	29.3	31.1	32.4	35.9	38	39.6
MLSS and MLVSS (mg L^{-1})	See table								See table

[1] Average value of the concentration present in the synthetic substrate and and liquid phase of te sludge sample prior the start of the test

MLSS & MLVSS measurements

Sampling point	Cup No.	W1	W2	W3	W2-W1	W2-W3	MLSS	MLVSS	Ratio
Start test[2]	1	0.09440	0.14430	0.09880	0.04990	0.04550	2,495	2,275	0.91
	2	0.09480	0.14210	0.09950	0.04730	0.04260	2,365	2,130	0.90
	3	0.09530	0.14190	0.09860	0.04660	0.04330	2,330	2,165	0.93
						Average	2,397	2,190	0.91
End test	4	0.09350	0.14360	0.09790	0.05010	0.04570	2,505	2,285	0.91
	5	0.09410	0.14330	0.09840	0.04920	0.04490	2,460	2,245	0.91
	6	0.09370	0.14200	0.09850	0.04830	0.04350	2,415	2,175	0.90
						Average	2,460	2,235	0.91

[2] Sample taken before substrate addition

Biomass composition

Sampling point	Start test	End test
MLSS (mg L^{-1})	2,397	2,460
MLVSS (mg L^{-1})	2,190	2,235
Ratio	0.91	0.91
Ash (mg L^{-1})	207	225

In this example, the observed $Y_{NH4\_NO2,AMX}$ and $Y_{NH4\_NO3,AMX}$ ratios are 1.22 and 0.24 g N g N^{-1}, respectively. The obtained specific biomass nitrogen removal rate is comparable with the kinetics reported in Table 2.4.3, indicating a high enrichment of anammox bacteria in the sludge sample tested. Also, the stoichiometric parameters of interest are in good agreement with the $Y_{NH4\_NO2,AMX}$ and $Y_{NH4\_NO3,AMX}$ reported in Table 2.4.3, indicating that the anaerobic ammonium oxidation is the dominant bioprocess occurring in the sludge sample analysed. It is important to note that when sludge samples containing COD are

analysed, the $Y_{NH4\_NO2,AMX}$ and/or $Y_{NH4\_NO3,AMX}$ are expected to be different from the reference stoichiometric values due to the simultaneous occurrence of conventional heterotrophic denitrification.

2.4.11 Additional considerations

2.4.11.1 Presence of other organisms

The presence of other microorganism rather than those that carry out the biological conversions under examination may alter the results of the batch tests resulting in the under- or over-estimation of kinetics and stoichiometric parameters of interest.

In nitrification batch activity tests, the simultaneous occurrence of denitrifying and/or anammox activity may lead to an incorrect estimation of nitrite removal and/or nitrate production. Nevertheless, since both biological processes require anoxic conditions, it is sufficient to ensure the complete penetration of oxygen into the biomass. While 3-4 mg O_2 L^{-1} are considered sufficient to ensure fully aerobic conditions when testing flocculent and suspended sludge samples, higher oxygen concentrations may be required when assessing the activity of nitrifying biofilms. It is important to note that when a batch test is performed in the presence of COD, the aerobic COD removal performed by heterotrophic microorganisms would further reduce the oxygen penetration into the biofilm, thus contributing to the creation of undesirable anoxic zones (if oxygen becomes limiting).

In denitrification batch activity tests, the simultaneous occurrence of anammox activity may lead to the overestimation of nitrite and/or underestimation of the nitrate reduction kinetics. In order to avoid this inconvenience, the batch test should be carried out under ammonium-limiting conditions. It is important to note that the ammonium present during the batch activity test should be sufficient anyway to sustain the N-source requirements of denitrifying bacteria, which can be calculated in advance. When the batch test aims to assess the impact of a particular carbon source on the denitrifying kinetics, the simultaneous occurrence of the denitrification process carried out by microorganisms that can store COD intracellularly (e.g. PAO and GAO) may lead to incorrect observations. In this case, an aeration period prior to the conduction of the batch test can be used to completely remove the intracellular stored COD present in the biomass.

In anammox batch activity tests, the simultaneous occurrence of denitrifying and anammox activity may lead to an incorrect estimation of the nitrite removal and/or nitrate production kinetics, which consequently affects the $Y_{NH4\_NO2,AMX}$ and/or $Y_{NH4\_NO3,AMX}$. When OHO are the dominant denitrifying population, it is sufficient to execute the batch test in the absence of S_B to limit the presence of electron-donating compounds used for nitrite/nitrate reduction. However, when EBPR activated sludge is used instead, this would not be sufficient since intracellular storage compounds would still allow the reduction of nitrite/nitrate during the anammox batch test. A period of aeration prior to the conduction of the anammox batch test can be used to completely remove the S_B in the bulk and/or the intracellular stored COD present in the activated sludge to be tested. Nevertheless, since the anammox pathway is the only one capable of oxidizing ammonium under anoxic conditions, anammox activity can be satisfactorily assessed in a batch test even in the presence of COD by following the ammonium concentration over time.

2.4.11.2 Shortage of essential micro- and macro-nutrients

Though it may seem trivial, the presence of macro- and micro-nutrients in the right concentration and (bio-) availability is essential for the bioprocesses involved in the nitrogen removal of activated sludge systems such as the nitrification, denitrification and anammox processes described in this chapter. Commonly, macro- and micro-nutrients are present in most municipal wastewaters, but their presence should be checked and confirmed particularly if the wastewater treatment plant under examination regularly receives industrial effluents. Due to the low yield of autotrophic microorganisms such as nitrifiers and anammox bacteria, the lack of macro-nutrients is not so frequent when treating municipal sewage. Nevertheless, it should be a point of attention when these bioprocesses are applied to the treatment of industrial wastewaters and in general for high-strength wastewaters. For this purpose, a simple estimation of the nutrient requirements of the biomass as a function of the nitrogen load to be treated can be performed and compared against the influent nutrient concentrations to assess whether external addition is necessary to support the biological growth requirements and conversion rate. In some cases the external dosage of particular micro-nutrients beyond the minimal requirements can enhance the kinetics of a specific microbial population as recently reported in the case of iron dosage to anammox cultures (Chen *et al.,* 2014; Bi *et al.,* 2014).

2.4.11.3 Toxicity or inhibition effects

Several compounds were identified to be toxic or inhibitory for the bacterial communities carrying out the nitrification, denitrification and anammox processes. Since the abundance of OHOs capable of performing denitrification in activated sludge systems is broad and diverse they can acclimatise and adapt to different environmental and operating conditions and even withstand the presence of different potentially toxic or inhibitory compounds. On the other hand, autotrophic bacteria catalysing the nitrification and anammox processes are more prone to inhibition and toxicity issues. The list of compounds and concentration ranges which may be toxic or inhibitory for nitrifying and anammox bacteria is so long and complex that one should refer to specific literature. Despite the fact that microbial communities can also acclimatise and adapt to sub-optimal conditions, the inhibitory effects of certain compounds can lead to sub-optimal microbial process activity and ultimately to the failure of the bioprocess. To assess the inhibitory effect of a certain compound or of a certain particular wastewater on the nitrifying, denitrifying or anammox activity, a series of batch activity tests can be conducted as described in this chapter. The comparison between the activity measured in the presence or absence of different concentrations of potential inhibitory compounds can provide useful indications about the inhibitory potential of such compounds with regard to the particular activated sludge tested. Similarly, when the wastewater used to perform the tests is suspected to have or to generate an inhibitory effect on a particular bioprocess, two series of batch activity tests can be executed: one with the original activated sludge and another one with the same biomass but washed in a mineral solution to remove the potentially inhibiting or toxic compounds. However, it is important to note that such an approach may be successful only if the inhibiting effects of the wastewater to be tested are (rapidly) reversible.

2.4.11.4 Effects of carbon source on denitrification

It is well known that denitrification kinetics depend on the carbon sources used as electron donors (e.g. Mokhayeri *et al.,* 2006, 2008). For the regular monitoring of the denitrifying potential of activated sludge, the use of synthetic media containing S_B such as VFAs can be good enough to provide a satisfactory assessment of denitrification activity (as presented in this chapter). The use and application of more complex COD sources, which may be undoubtedly present in raw or settled municipal wastewater, can lead to sub-optimal denitrification activity. When external COD sources are needed to enhance nitrogen removal (e.g. in the post-denitrification unit), the methods described in this chapter can be used to assess the influence of different COD sources on the denitrifying kinetics of an activated sludge sample.

Figure 2.4.15 Execution of batch activity tests. Note the characteristic reddish colour of the enriched anammox culture (photo: Lotti, 2015).

2.5 AEROBIC ORGANIC MATTER REMOVAL

2.5.1 Process description

In conventional wastewater treatment systems performing aerobic organic matter removal, OHOs remove the organics present in wastewater to produce more biomass using oxygen for respiration. From a metabolic perspective, the removal process involves an anabolic (for cell synthesis) and a catabolic process (to generate the required energy for cell synthesis). In the anabolic process, OHOs obtain the required carbon for cell growth from the organic matter present in wastewater. Meanwhile, in the catabolic process, an oxidation-reduction reaction takes place involving the transfer of electrons from the organic matter (which acts as the electron donor) to oxygen (the electron acceptor), generating the required energy for cell synthesis. However, due to the rather variable mixture of biodegradable and non-biodegradable organic compounds present in wastewater, the COD is commonly used to estimate their total concentration. This is mostly because the use of COD is preferred over other analytical parameters (such as biochemical oxygen demand: BOD_5 or total organic carbon: TOC) due to several advantages. These include (Henze *et al.,* 1997; Henze and Comeau, 2008): (*i*) the determination of the oxygen equivalence (or capacity to donate electrons) of the organic compounds, (*ii*) the more detailed and useful determination of the organic strength by being able to measure all the degradable and undegradable organics, (*iii*) the potential for the balance of organics to be closed on a COD basis (as a consequence of the previous two advantages), as well as practical implications such as (*iv*) a rapid analysis (i.e. a few hours as opposed to 5 days required for BOD_5). In general, the exact stoichiometry involved in the aerobic removal of organics is not straight forward. Nevertheless, the following equation for the aerobic consumption of glucose ($C_6H_{12}O_6$) (which neglects most of the nutrients except nitrogen) can be used to illustrate the biological aerobic removal process (Metcalf and Eddy, 2003):

$$3C_6H_{12}O_6 + 8O_2 + 2NH_3 \rightarrow 2C_5H_7NO_2 + 8CO_2 + 14H_2O$$

Eq. 2.5.1

Where, $C_5H_7NO_2$ is a simplified expression of the new cells generated from the aerobic degradation of organics (Hoover and Porges, 1952).

From a microbial growth perspective, about ⅔ of the biodegradable organics (leading to the so-called aerobic stoichiometric true yield, Y_{OHO}, of ~0.67 g COD-biomass per COD-organics consumed) are converted into new biomass via anabolism and the remaining ⅓ is oxidized using oxygen via catabolic pathways to generate the required energy for biomass growth (Marais and Ekama, 1976). In addition, for microbial growth, macronutrients (like nitrogen and phosphorus) and micronutrients (such as potassium, sodium, calcium, magnesium, zinc, manganese and iron, among others) are needed for cell synthesis (Metcalf and Eddy, 2003). The lack of any of these elements can lead to limitations of the microbial processes. It is assumed that the nitrogen and phosphorus requirements of the new biomass produced are approximately 0.10 g N g VSS^{-1} and 0.03 g P g VSS^{-1}, respectively (Ekama and Wentzel, 2008a). This means that if a wastewater contains 100 mg BCOD L^{-1} (biodegradable organics) then the nitrogen and phosphorus concentrations that need to be supplied should not be lower than 4.7 mg NH_4-N L^{-1} and 1.4 mg PO_4-P L^{-1}, respectively, to meet the nutrient requirements. This assumes a true yield of Y_{OHO} of 0.67, an observed yield of 0.40 (accounting for a sludge age of about 5 days) and a COD-to-VSS ratio of the biomass of 1.42 mg COD mg VSS^{-1}. From a practical perspective and to avoid nutrient limitations due to the different Y_{OHO} observed (see Table 2.5.1), a COD:N:P ratio of 100:5:1 is usually suggested (Metcalf and Eddy, 2003).

It is important to underline that not all the organics present in wastewater can be subject to degradation. At the most basic classification, at least four different organic fractions can be identified in a wastewater stream with different physical characteristics and degree of biodegradability that determine their removal potential in a wastewater treatment system (Ekama and Wentzel, 2008a). These are (*i*) biodegradable soluble organics rapidly converted by OHO (and thus known as readily biodegradable organics, RBCOD or S_B according to the standardized notation (Corominas *et al*., 2010)), (*ii*) biodegradable particulate organics that get mostly enmeshed within the activated sludge flocs and are subject to hydrolysis prior to biodegradation, and therefore commonly characterized as slowly biodegradable organics (SBCOD or XC_B in accordance with Corominas *et al*., 2010), (*iii*) non-biodegradable particulate organics that get mostly enmeshed in the sludge flocs and accumulate in the activated sludge system, and (*iv*) non-biodegradable soluble organics which neither get enmeshed nor degraded and remain in the soluble phase.

RBCOD can be rapidly utilized by OHOs. Although the biological breakdown of SBCOD is slow, at the SRT commonly applied in most activated sludge plants (e.g. longer than 3-4 days), SBCOD are virtually completely utilized (Ekama and Wentzel, 2008a). Thus, OHOs utilize RBCOD and SBCOD for cell synthesis, producing more biomass. The new OHO biomass generated by the removal and conversion of RBCOD and SBCOD, as well as the accumulation of the non-biodegradable particulate organics, becomes part of the organic activated sludge mass in the reactor usually measured as MLVSS. Because of the flocculation capability of the activated sludge, solids material is relatively highly settleable so it can be efficiently removed in the secondary settling tanks, providing a treated and clear effluent. The sludge mass that settles out in the secondary settling tank is returned to the biological reactor and eventually is removed via the waste of activated sludge which is a function of the plant's SRT (Arden and Lockett, 1914). However, since the non-biodegradable soluble organics cannot be efficiently removed in an activated sludge system, they leave the plant through the effluent, contributing to the effluent COD concentration.

In addition to the aerobic removal of organics, in activated sludge systems performing BNR, most of the organic matter will be removed in the anaerobic and anoxic stage that precedes the aerobic stage. For instance, in the anaerobic stage of an activated sludge system designed for EBPR, VFA are taken up by PAO, while in the anoxic stage of a BNR plant, biodegradable organics are used by denitrifying organisms for denitrification purposes using nitrate or nitrite as the electron acceptor. In addition, the potential occurrence of sulphate-reducing processes by SRBs in the anaerobic stages of an activated sludge system can also lead to the removal of organics. Furthermore, the removal of organics can also take place in anaerobic wastewater treatment systems (e.g. upflow anaerobic sludge blankets: UASB plants) under fully anaerobic conditions by strictly anaerobic organisms.

As observed, organic matter removal can occur under different environmental conditions and be performed by different groups of microorganisms. The present section focuses on the execution of batch activity tests to assess aerobic organic matter removal as the primary removal process by OHOs in conventional activated sludge systems under fully aerobic conditions. The activity assessment of other processes is presented in other sections of the present and following chapters.

The present section aims to be used as a guide for the execution of aerobic batch activity tests for the determination of the kinetic rates of RBCOD removal in activated sludge and other suspended growth systems. It does not cover the removal of SBCOD fractions because its determination requires the execution of respirometry tests described in Chapter 3. Moreover, and despite that the fractionation of the influent wastewater organic compounds into COD (as well as into N and P) is of major importance for the design, operation, modelling and evaluation of (activated sludge) wastewater treatment plants, the current chapter does not aim to elaborate on wastewater characterization and fractionation protocols. For such a purpose, the reader is referred to scientific and technical reports published elsewhere (Henze, 1992; Kappeler and Gujer, 1992; Wentzel *et al.,* 1995; Hulsbeek *et al.,* 2002; Roeleveld and van Loosdrecht, 2002; Vanrolleghem *et al.,* 2003; WERF, 2003; Langergraber *et al.,* 2004).

It is important to note that some of the wastewater characterization and fractionation protocols, as well as a thorough determination of the actual biomass yields using organics from different wastewaters, also need respirometry tests (Chapter 3).

2.5.2 Experimental setup

2.5.2.1 Reactors

To assess the aerobic organic matter removal activity by activated sludge, batch tests need to be carried out under aerobic conditions securing sufficient availability of DO (maintaining DO concentrations higher than 2 mg L^{-1}) and good mixing conditions. As for other processes, it is also important to maintain an adequate and desirable temperature, precise pH control, and have additional ports for sample collection and the addition of influent, solutions, gases and any other liquid media or substrate used in the test. In general, similar fermenters with features and characteristics of those used for the execution of EBPR batch activity tests can be used to carry out the aerobic organic matter removal tests (see Section 2.2.2.1). Similar recommendations to those described in Section 2.2.2.1 regarding aeration, mixing and pH control, location and characteristics of sampling and dosing ports can also be applied here.

2.5.2.2 Activated sludge sample collection

For the execution of aerobic organic matter removal batch activity tests, a fresh sample should be collected at the end of the aerobic tank or phase in a sampling spot where well-mixed conditions take place. Ideally, batch

activity tests should be performed soon after collection (within 2 to 3 h after sampling). If the batch activity tests cannot be performed *in situ* on the same day when sampling took place, an activated sludge sample can be collected in a bucket or jerry can at the end of the aerobic stage and properly transported and stored in a fridge or using ice (to keep the temperature around 4 °C if feasible). In any case, the *in situ* execution of the batch activity tests is preferable for obvious reasons. The total volume of activated sludge to be collected depends on the number (repetition) of tests, reactor volume and total volume of samples to be collected to assess the biomass activity. Often, 10-20 L of activated sludge collected from full-scale wastewater treatment plants can be considered sufficient per batch. On the other hand, samples collected from lab-scale reactors rarely provide more than 1 L because lab-scale systems use small reactors (from 0.5 to 2.4 L and in some cases up to 8-10 L, exceptionally 15 L) and the maximum volume that can be withdrawn from lab-scale reactors is often set by the daily withdrawal of the excess of sludge from the system (which is directly related to the SRT and, consequently, defined by the growth rate of the organism(s) of interest).

2.5.2.3 Activated sludge sample preparation

For batch activity tests performed *in situ*, in principle, the sludge should be transferred from the parent reactor (in the case of a lab-enriched sludge) or reaction tank (in the case of pilot-scale or full-scale plants) to the fermenter or reactor where the activity tests will take place. Then the activated sludge should be aerated for at least 1 or 2 h to remove any residual biodegradable COD present in the system while the sample is also adjusted to the desired pH and temperature of interest (as described in Section 2.2.3.5). Alternatively, to remove any residual COD the activated sludge can be washed using the washing media and washing procedure described in sections 2.2.3.3 and 2.2.3.5, respectively.

If only an aerobic organic removal batch activity test is going to be executed (i.e. a nitrification test is not of interest), then a nitrification inhibitor can be added to the sludge sample immediately after the sludge has been transferred to the fermenter (e.g. allyl-N-thiourea: ATU to a recommended final concentration of 20 mg L^{-1}). In particular, this will restrain nitrification and consequently avoid higher oxygen consumption if respirometry tests are going to be executed in parallel (see Chapter 3). Sludge samples stored under cold conditions can also be washed with a mineral solution to remove any residual organics.

For batch tests executed with sludge samples stored under cold conditions (at around 4 °C), sludge samples need to be 're-activated' because the cold storage temperature slows down the bacterial metabolism. To re-activate the sludge, the activated sludge should be aerated for 1-2 h at the desired pH and, particularly, the temperature of study.

2.5.2.4 Media

When real wastewater (either raw or settled) is used for the execution of activity tests, it can be fed in a relatively straightforward manner to the reactor/fermenter. For normal or regular conditions, the feeding step takes place at the beginning of the test. If required, raw wastewater can be filtered (using 1 or 2 mm sieves) or settled (duration from 1 to 3 h). If the activity tests need to be performed at different solids concentration, (*i*) the treated effluent from the plant can be collected and used for dilution (assuming that solids effluent concentrations are relatively low, e.g. 20-30 mg TSS L^{-1}), or (*ii*) the activated sludge can be concentrated by decanting and discharging the supernatant in several repeated steps until reaching the MLSS of interest.

If different carbon sources and concentrations are to be studied, the plant effluent can also be used to prepare a semi-synthetic media (as long as it does not contain toxic or inhibitory compounds) containing a RBCOD concentration of interest which, for instance after a 1:1 dilution in the fermenter, can provide the target initial COD concentration. However, depending upon the nature and purpose of tests, the carbon concentrations present in a synthetic wastewater can vary and be adjusted proportionally to the duration of the test. Usually, concentrations of about 400 mg COD L^{-1} can be used with either lab- or full-scale activated sludge. Besides the carbon source, the synthetic wastewater must contain the required macro- and micro-elements. A suggested synthetic wastewater recipe for an initial COD of about 400 mg COD L^{-1} can contain per litre (Smolders *et al.,* 1994a): 862 mg $NaAc \cdot 3H_2O$ (400 mg COD), 107 mg NH_4Cl (28 mg N), 40 mg $NaH_2PO_4 \cdot 2H_2O$ (8 mg P), 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 gEDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 g H_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). If desired, the synthetic wastewater can be concentrated to a higher desirable COD concentration to account for potential dilution rates, sterilized in an autoclave (for 1 h at 110 °C) and used as

a stock solution if several tests are going to be performed in a defined period of time. However, the solution must be discarded if any precipitation is observed or it loses transparency.

For experiments performed with lab-enriched cultures, it is best to execute the tests with the same (synthetic) wastewater used for the cultivation with the carbon concentrations of study, unless otherwise required. Alternatively, and similar to full-scale samples, the effluent from the reactor can be collected, filtered through rough pore size filters to remove any coarse particles, and used to prepare the required media.

2.5.2.5 Analytical tests

Most of the analytical tests required (for the determination of COD, total P, PO_4, NH_4 NO_2, NO_3, TSS, VSS, etc.) can be performed following standardized and commonly applied analytical protocols detailed in Standard Methods (APHA *et al.,* 2012). For the determination of dissolved parameters like soluble COD, PO_4, NH_4, NO_2 and NO_3, samples should be filtered immediately after collection through 0.45 µm pore size filters. Of the two most commonly applied methods for the analytical determination of COD, the dichromate method is recommended, as the permanganate method does not fully oxidize all the organic compounds (Henze and Comeau, 2008). The determination of VFA (like acetate, propionate and other volatile fatty acids) can be executed by GC. Glucose and other carbon compounds (including VFA) can be determined by HPLC.

2.5.2.6 Parameters of interest

To determine and assess the activity of OHOs, different stoichiometric ratios and kinetic rates can be estimated as displayed in Table 2.5.1.

Table 2.5.1 Expected stoichiometric and kinetic parameters of interest for activated sludge systems performing aerobic organic matter removal.

Parameter	Remark	Reference
Aerobic stoichiometric parameter Y_{OHO} (g COD-biomass g COD-substrate^{-1})		
0.67	Theoretical ratio	Ekama and Wentzel (2008a)
0.72	Acetate as organic matter source	Dircks *et al.* (1999)
0.37	Methanol as organic matter source	McCarty (2007)
0.65	Formate as organic matter source	McCarty (2007)
0.40 -0.80; typically 0.60	g VSS g BOD^{-1} units	Metcalf and Eddy (2003)
0.30-0.60	g VSS g RBCOD^{-1} units	Metcalf and Eddy (2003)
0.67-0.792*	Different RBCOD organic matter sources	Guisasola (2005)
0.91*	Glucose as organic matter source	Dircks *et al.* (1999)
0.90*	Glucose as organic matter source	Goel *et al.* (1999)
Aerobic kinetic parameter $q_{OHO,COD,Ox}$ (g COD substrate g COD-biomass^{-1} d^{-1})		
6	ASM2d model	Henze *et al.* (1999)
2-10, typically 5	g RBCOD g VSS^{-1} d^{-1}	Metcalf and Eddy (2003)
3 - 10		Kappeler and Gujer (1992)

* These values deviate considerably from the theoretical yield of 0.67 gCOD gCOD^{-1} due to the occurrence of storage processes.

It is important to mention that an Arrhenius temperature coefficient of between 1.060 and 1.123 (with a typical value of 1.070) has been suggested for the description of the aerobic organic matter removal rates (Metcalf and Eddy, 2003).

As shown in Table 2.5.1, the theoretical stoichiometric biomass yield on biodegradable organics is 0.67 g COD g COD^{-1} (Metcalf and Eddy, 2003; Ekama and Wentzel, 2008a). However, it is common to observe higher stoichiometric ratios that can apparently reach up to 0.90-0.91 g COD g COD^{-1} (Dircks *et al*., 1999; Goel *et al*., 1999). If this is the case, one should be aware that values higher than 0.67 g COD g COD^{-1} are caused by the direct storage of biodegradable organics rather than a higher biomass yield. Such processes usually occur when RBCOD (S_B) is the dominant organics in plants designed with selectors. Further details can be found in literature (Gujer *et al*., 1993; Henze *et al*., 2008). Regarding the aerobic kinetic rates for organic matter removal, those

reported in literature can vary widely from 3 to 10 g COD-substrate g COD-biomass^{-1} d^{-1}. The main reason for this can be the net concentration of active biomass present in the system (with respect to the total MLVSS concentration). A short SRT (of less than 3 days) may lead to a high fraction of active biomass with respect to VSS present in the system which could be reflected in a high organic matter removal rate. However, a (very) long SRT (for instance, much higher than 20 days) will lead to a higher accumulation of non-biodegradable VSS (present in the influent) or produced by endogenous respiration by OHOs (also measurable as MLVSS) which adds to the MLVSS concentration of the sludge and leads to a lower maximum specific COD removal rate, $q_{OHO,COD,Ox}$. Thus, the highest $q_{OHO,COD,Ox}$ (up to 10 g COD-substrate g COD-biomass^{-1} d^{-1}) can be expected in tests executed with activated sludge samples containing relatively higher concentrations of active OHO biomass, typically observed in low sludge age systems.

2.5.3 Aerobic organic matter batch activity tests: preparation

2.5.3.1 Apparatus

For the execution of aerobic organic batch activity tests the following apparatus is needed:

1. A batch reactor or fermenter equipped with a mixing system and adequate sampling ports (as described in Section 2.5.2.1).
2. An oxygen supply (compressed air or pure oxygen sources).
3. A pH electrode (if not included/incorporated in the batch reactor setup).
4. A 2-way pH controller for HCl and NaOH addition (alternatively a one-way control - generally for HCl addition - or manual pH control can be applied through the manual addition of HCl and NaOH).
5. A thermometer (recommended working temperature range of 0 to 40 °C).
6. A temperature control system (if not included in the batch reactor setup).
7. A DO meter with an electrode (if not included/incorporated in the batch reactor setup).
8. An automatic 2-way controller for nitrogen and oxygen gas supply (if not included in the batch reactor setup and if tests must be performed at a defined DO concentration).
9. A centrifuge with a working volume capacity of at least 250 mL to carry out the sludge washing procedure (if required).
10. A stopwatch.

Confirm that all the electrodes and meters (pH, temperature and DO) are calibrated less than 24 h before execution of the batch activity tests in accordance with guidelines and recommendations from manufacturers and/or suppliers.

2.5.3.2 Materials

1. Two graduate cylinders of 1 or 2 L (depending upon the sludge volumes used) to hold the activated sludge and wash the sludge if required.
2. At least 2 plastic syringes (preferably of 20 mL or at least of 10 mL volume) for the collection and determination of soluble compounds (after filtration).
3. At least 3 plastic syringes (preferably of 20 mL) for the collection of solids, particulate or intracellular compounds (without filtration).
4. 0.45 µm pore size filters. Preferably not of cellulose-acetate because these may release certain traces of cellulose or acetate into the collected water samples. Consider using twice as many filters as the number of samples that need to be filtered for the determination of soluble compounds.
5. 10 or 20 mL transparent plastic cups to collect the samples for the determination of soluble compounds (e.g. soluble COD, ammonium, ortho-phosphate).
6. 10 or 20 mL transparent plastic cups to collect the samples for the determination of mixed liquor suspended solids and volatile suspended solids (MLSS and MLVSS, respectively). Consider the collection of these samples in triplicate due to the variability of the analytical technique.
7. A plastic box or dry ice box filled with ice up to the required volume to temporarily store (for up to 1-2 h after the conclusion of the batch activity test) the plastic cups and plastic tubes for centrifugation after the collection of the samples.
8. Plastic gloves and safety glasses.
9. Pasteur or plastic pipettes for HCl and/or NaOH addition (when the pH control is carried out manually).
10. Metallic lab clips or clamps to close the tubing used as a sampling port when samples are not collected from the reactor/fermenter.

2.5.3.3 Working solutions

- **Real wastewater**

  If real wastewater is used to carry out the batch activity test, there is a need to collect the sample at the influent of the wastewater treatment plant and

perform the batch activity test as soon as possible after collection. If due to location and distance the tests cannot be performed in less than 1 or 2 h after collection, then one should keep the wastewater sample cold until the test is executed (e.g. by placing the bucket or jerry can in a fridge at 4 °C). Nevertheless, prior to the execution of the test, the temperature of wastewater needs to be adjusted to the target temperature at which the batch activity test will be executed (preferably reached in less than 1 h). A water bath or a temperature-controlled room can be used for this purpose.

- **Synthetic influent media or substrate**

  If tests can be or are desired to be performed with synthetic wastewater, then the synthetic influent can contain a mixture of carbon and (macro- and micro-) nutrients. Generally, they can all be mixed together in the same media, as long as precipitation is not observed. The usual composition and concentration are:

  a. Carbon source solution: this must be composed of a RBCOD source like VFA (such as acetate or propionate) or glucose, depending on the nature or goal of the test and the corresponding research questions. Initial COD concentration of around 400 mg L^{-1} is recommended for sludge samples from either lab- or full-scale systems.
  b. The nutrient-solution: this should contain all the required macro- (ammonium, phosphorus, magnesium, sulphate, calcium, potassium) and micro-nutrients (iron, boron, copper, manganese, molybdate, zinc, iodine, cobalt) to ensure that cells are not limited by their absence and avoid obtaining erroneous results and in extreme cases the failure of the test. Thus, although their concentrations may seem trivial, one must make sure that all of the constituents are added to the solution in the required amounts. The following composition for a nutrient solution can be recommended for influents containing up to 400 mg COD L^{-1} (based on Smolders *et al.,* 1994a) per litre: 107 mg NH_4Cl (28 mg), 90 mg $MgSO_4 \cdot 7H_2O$, 40 mg $NaH_2PO_4 \cdot 2H_2O$ (8 mg P), 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 gH_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). Similar nutrient solutions can be used as long as they contain all the previously reported required nutrients.
  c. Washing media: If the sludge sample must be washed to remove any residual COD or the presence of undesirable compounds (which may be even inhibitory or toxic), a nutrient solution should be prepared. The following nutrient solution to wash the sludge could be used per litre (Smolders *et al.,* 1994a): 107 mg NH_4Cl, 40 mg $NaH_2PO_4 \cdot 2H_2O$, 90 mg $MgSO_4 \cdot 7H_2O$, 14 mg $CaCl_2 \cdot 2H_2O$, 36 mg KCl, 1 mg yeast extract and 0.3 mL of a trace element solution (that includes per litre 10 g EDTA, 1.5 g $FeCl_3 \cdot 6H_2O$, 0.15 gH_3BO_3, 0.03 g $CuSO_4 \cdot 5H_2O$, 0.12 g $MnCl_2 \cdot 4H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$, 0.12 g $ZnSO_4 \cdot 7H_2O$, 0.18 g KI and 0.15 g $CoCl \cdot 6H_2O$). The washing process can be repeated two or three times. Afterwards, the following preparation steps of the batch activity tests can be performed.
  d. ATU solution: To inhibit nitrification, an ATU solution can be prepared to reach an initial concentration of around 20 mg L^{-1} (after addition). The ATU solution must be added before the sludge is exposed to any aerobic conditions (including the sludge sample preparation or acclimatization).
  e. Acid and base solutions: 100-250 mL of 0.2 M HCl and 100-250 mL 0.2 M NaOH solutions for automatic or manual pH control.

Finally, the required working and stock solutions to carry out the determination of the analytical parameters of interest must be also prepared in accordance with Standard Methods and the corresponding protocols.

2.5.3.4 Material preparation

1. Collect the materials to execute the batch activity tests and the definition of the number of samples to be collected can be prepared following the same steps presented in Section 2.2.3.4.
2. Determine the frequency of sample collection: for the determination of the maximum RBCOD kinetic removal rate, samples need to be collected every 5 min during the first 30-40 min of duration of the batch activity test.
3. Collect a series of samples before any media is added to increase the data reliability and to establish the initial conditions of the sludge.
4. Carefully define the maximum and minimum working volumes of the reactor according to the suggestions presented in Section 2.2.3.4.

5. Label all the plastic cups once the number and frequency of collecting the samples has been defined. Define a nomenclature and/or abbreviation to easily identify and recognize the batch test, the sampling time and the parameter(s) of interest to be determined with that sample. Labelling both the plastic cup and the cover will help to easily identify the sample.
6. Table 2.5.2 contains an example of a working sheet to execute and keep track of the sampling collection and batch test execution. Furthermore, it can be used to keep a database of the different batch tests carried out.
7. Organize all the required material within a relatively close radius of the action around the batch setup so any delay in handling and preparing the samples can be avoided.
8. Calibrate all the meters (pH, DO and thermometer) less than 24 h prior to the execution of the tests and store them in proper solutions until the execution of the tests, according to the particular recommendations of the corresponding manufacturer or supplier. Also confirm that the readings are reliable.
9. Store the samples properly and preserve them until they are analysed. Table 2.2.2 provides recommendations for sample preservation depending on the analytical determination of the parameter of interest.

2.5.3.5 Activated sludge preparation

These procedures consider that batch activity tests can be performed as soon as possible after collection of samples from full- or lab-scale systems or, in the worst case scenario, within 24 h after collection. Ideally, the execution of batch tests 24 h after the collection is not recommended due to potential changes and decay of the microorganisms. Bearing this in mind, the following procedure is recommended to prepare the activated sludge samples for the execution of batch activity tests:

1. If batch activity tests can be executed in less than 1 h after collection of the sludge sample and if the sludge sample does not need to be washed:
   a. Adjust the temperature of the batch reactor to the target temperature of the study.
   b. Collect the sludge at the end of the aerobic stage.
   c. Transfer the sludge sample to the reactor or fermenter.
   d. Add the ATU solution to inhibit nitrification (particularly if respirometry tests will be executed) to a final concentration of 20 mg L^{-1} (see Section 2.5.3.3).
   e. Start mixing (100-300 rpm) and follow the temperature and pH of the sludge sample by placing an external thermometer inside the reactor (if the setup does not have a built-in thermometer) until the sludge reaches the target temperature and pH of the study.
   f. Start to aerate the sludge samples keeping a DO concentration higher than 2 mg L^{-1}.
   g. Start the aerobic batch activity tests once the pH and DO are stable.
2. If the activated sludge sample needs to be washed then the steps presented in Section 2.2.3.5 (for tests that can be executed in less than 1 h or 24 h after collection) can be followed for the activated sludge sample preparation using the washing media indicated in Section 2.5.3.3.

2.5.4 Aerobic organic matter batch activity tests: execution

Similar to previous tests, to facilitate the execution and for data track record and archiving purposes, an experimental implementation plan should be prepared in advance similar to that presented in Table 2.5.2. Aerobic organic matter removal activity tests can be executed to assess the removal of RBCOD in activated sludge systems. They can be performed with activated sludge samples from full- or lab-scale systems, using real or synthetic wastewater. Samples should be collected at the end of the aerobic stage as long as RBCOD is not present in the sample. The following steps are proposed for the execution of the aerobic organic matter removal test:

Test OHO.AER.1. Single aerobic organic matter removal test

a. After the sludge has been collected, prepared and transferred to the batch reactor (see Section 2.5.3.5), keep the sample aerated for at least 30 min while confirming that the pH and temperature are at the target values of interest. Otherwise, set up the corresponding set points (if automatic pH and temperature controllers are applied) or adjust manually. Wait until stable conditions are reached.
b. Ensure that the DO concentration is higher than 2 mg L^{-1}.
c. Keep track of the execution and sampling time with a stopwatch.
d. Take the first samples of the water phase and biomass once stable operating conditions are reached (around 20 min before the start of the test) to determine the initial concentrations of the parameters of interest:

soluble COD, NH_4, PO_4 and MLSS and MLVSS concentrations.

e. Connect the syringe to take the samples, next open or release the lab clip or clamp that closes the sampling port, and then pull and push the syringe several times until a homogenous sample is collected (usually around 5 times are required). When the syringe is full, close the clip and remove the syringe.
f. Immediately filter the samples used for the determination of soluble COD, NH_4, PO_4 (through 0.45 μm pore size filters). Other samples (e.g. MLVSS and MLVSS) need to be prepared in accordance with the corresponding protocols.
g. Store the samples at 4 °C in the fridge or preferably in a cool box with ice before and during the test execution.
h. Start the execution of the aerobic test at 'time zero' with the addition of the real or synthetic wastewater (as the carbon source solution).
i. Add the real wastewater or synthetic influent to reach an initial RBCOD-to-MLVSS ratio in the reactor of around 0.10 mg COD mg VSS^{-1}. For example, the initial RBCOD and MLVSS concentrations after mixing can range around 400 mg COD L^{-1} for samples containing 4,000 mg VSS L^{-1}. Higher or lower ratios may also be acceptable as long as there is sufficient time for sampling.
j. Ensure that the DO readings remain above 2 mg L^{-1} after the addition of the wastewater, and that considerable temperature and pH variations (higher than 1 °C or ± 0.1 for temperature and pH, respectively) do not take place.
k. Duration and sampling:
   (i) Usually tests can last between 2 and 4 h for initial soluble COD concentrations of up to 400 mg COD L^{-1} (at pH 7.0 and 20 °C).
   (ii) To determine the aerobic kinetic parameters, samples for the determination of soluble COD should be collected every 5 min in the first 30-40 min of execution of the test. After this period, the sampling frequency can be reduced to 10 or 15 min during the first 1 h, and later on to every 15 or 30 min until the test is finished.
l. Conclude the aerobic test with the collection of samples for the determination of soluble COD, MLSS, MLVSS, NH_4 and PO_4 concentrations.
m. Organize the samples and ensure that all the samples are complete and properly labelled to avoid mixing the samples and trivial mistakes.
n. Preserve and store the samples as recommended by the corresponding analytical procedures until the collected samples are analysed.
o. Clean up the apparatus and take appropriate measures to maintain and look after the different sensors, equipment and materials.
p. Keep (part of) the sludge used in the test for possible further use (e.g. for microbial identification, see chapters 7 and 8).

2.5.5 Data analysis

The growth yield of biomass on COD is the main stoichiometric parameter of interest to assess the aerobic stoichiometry of OHO (Table 2.5.1). However, the aerobic batch activity test presented in this section cannot be used for the determination of Y_{OHO}. This is mostly because, for the determination of $Y_{OHO,}$ respirometry tests need to be executed in parallel to the aerobic tests to assess the amount of COD being used for energy generation. This is presented elsewhere (Wentzel *et al.,* 1995; Dircks *et al.,* 1999; Goel *et al.,* 1999; Guisasola *et al.,* 2005).

The maximum specific aerobic RBCOD removal rate ($q_{OHO,COD,Ox}$) can be computed by plotting the experimental data (Y-axis) versus time (X-axis) and fitting the experimental data obtained in the aerobic batch activity tests using linear regression. Because one is interested in the maximum rates, a linear regression approach can be applied by fitting more than 4 to 5 experimental data points while achieving a statistical determination coefficient (R^2) not lower than 0.90-0.95. This is the main reason why the sampling frequency within the first 30-40 min of execution of the batch activity tests is set to 5 min. The maximum volumetric kinetic rate can be determined based on the slope of the linear regression equation. This will result in the determination of the maximum volumetric rate (usually reported in units such as mg L^{-1} h^{-1} or g m^{-3} d^{-1}). Figure 2.5.1 illustrates an estimation of the maximum aerobic kinetic removal rates for an OHO culture. The rate can be expressed as the maximum specific kinetic rate by dividing the volumetric rate (or value of the slope) by the concentration of activated sludge VSS. It is important to note that the maximum biomass-specific growth rate cannot be computed in a straightforward manner by following the increase in biomass concentrations during the cycle since it may be practically negligible and fall into the standard error of the analytical determination of MLVSS. Instead, either long-term continuous tests can be executed or the aerobic activity tests need to be combined with respirometry tests (Chapter 3) as presented elsewhere in literature (Kappeler and Gujer, 1992; Wentzel *et al.,* 1995).

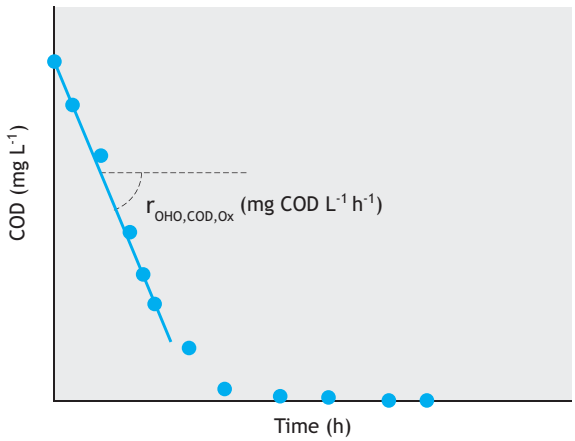

Figure 2.5.1 Example of the determination of the maximum aerobic volumetric kinetic rate for organic matter removal (expressed in mg COD L^{-1} h^{-1}) in an aerobic batch activity test.

2.5.6 Example

2.5.6.1 Description

To illustrate the execution of an aerobic batch activity test for OHO, data from a test performed at 15 °C with a full-scale activated sludge sample is presented in this section. The Test OHO.AER.1 was carried out to determine the aerobic kinetic rate of OHO for RBCOD removal. Thus, the batch activity test was performed using a 3.0 L reactor. All the equipment, apparatus and materials were prepared as described in Section 2.5.3. The pH and DO sensors were calibrated less than 24 h before the test execution. The test lasted 4 h. Prior to the batch test, 1.25 L of fresh activated sludge collected in the end of the aerobic phase of a full-scale plant was transferred to the reactor and acclimatized for 1 h at 15 °C under slow mixing (100 rpm) at pH 7.0 following the recommendations described in Section 2.5.3.5. Activated sludge preparation for tests was performed less than 1 h after sludge collection. Thereafter, 20 min before the start of the test, mixing was increased to 300 rpm and samples for the determination of the parameters of interest were collected (in accordance with the execution of Test OHO.AER.1). The test started with the addition of 1.25 L synthetic media containing 400 mg COD L^{-1} as Ac. Other macro- and micro-nutrients as well as 20 mg L^{-1} ATU were included in synthetic media in accordance with Section 2.5.3.3. Because the test was executed at 15 °C, the temperature of the synthetic media was also adjusted to 15 °C in a water bath operated at the same temperature before addition. Samples were collected every 5 min in the first 30 min of the aerobic phase. Immediately after collection, all the samples were prepared and preserved as described in Section 2.5.3.4. All the collected samples were analysed as described in Section 2.5.2.4. Table 2.5.2 shows the experimental implementation plan of the test.

2.5.6.2 Data analysis

Following the results of the batch activity test shown in Table 2.5.2, Figure 2.5.2 displays the estimation of the maximum volumetric OHO kinetic rates by applying linear regression.

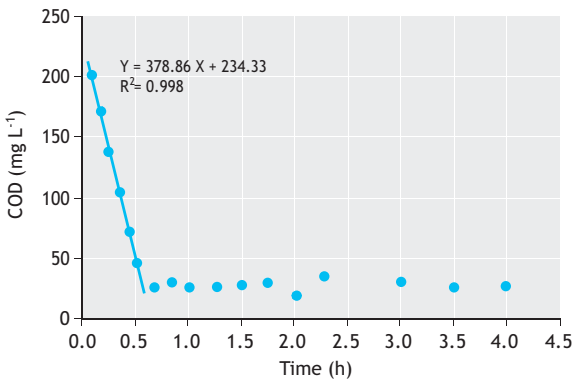

Figure 2.5.2 Graphic representation of the data obtained in the example of an experimental implementation plan for the execution of a batch activity test (Type OHO.AER.1) performed with a full-scale activated sludge at 15 °C using synthetic influent at pH 7.0. The main trend line shows the RBCOD conversion rate for the further estimation of the maximum specific kinetic rate.

Based on the maximum volumetric COD removal rate showed in Figure 2.5.2 and taking into account the MLVSS concentration of 1,890 mg L^{-1} (as an average MLVSS concentration observed between sludge samples collected at the beginning and end of the test), a specific RBCOD removal rate of 0.20 g COD g VSS^{-1} h^{-1} can be estimated, which corresponds to about 4.81 g RBCOD g VSS^{-1} d^{-1}. Using a COD-to-VSS ratio for the biomass of 1.42 g COD g VSS^{-1} (Metcalf and Eddy, 2003), a $q_{OHO,COD,Ox}$ of 3.39 g RBCOD g VSS^{-1} d^{-1} can be computed. Furthermore, since the test was not executed at 20 °C but at 15 °C, using a typical Arrhenius coefficient of 1.07 for OHO activity (Metcalf and Eddy, 2003), a $q_{OHO,COD,Ox}$ of 4.75 g RBCOD g COD^{-1} d^{-1} can be determined (3.39 g RBCOD g VSS^{-1} d^{-1} / $1.07^{(15-20)}$). This

value is in the range of other maximum specific kinetic rates reported for aerobic organic matter removal processes. It is important to note that the synthetic influent contained 400 mg COD L^{-1}, and the COD concentration measured 5 min after the start of the test was 203 mg COD L^{-1} due to the dilution of the 1.25 L of synthetic influent with the 1.25 L of activated sludge (which did not contain RBCOD) and the rapid kinetic removal rate of the biomass. Moreover, it can also be noticed that: (*i*) the initial COD concentration in the activated sludge prior to the wastewater addition was around 56 mg L^{-1} and, (*ii*) the final COD concentration at the end of the test remained at around 30 mg COD L^{-1}. The latter concentration corresponds to the soluble non-biodegradable COD present in the activated sludge sample which cannot be removed by biological means (as explained earlier in this chapter).

Table 2.5.2 Example of an experimental implementation plan for the execution of a batch activity test (Type OHO.AER.1) performed with a full-scale activated sludge at 15 °C using synthetic influent at pH 7.0.

Aerobic COD removal batch tests — **Code: OHO.AER.1**

Date:	Thursday 09.10.2015 10:00 h
Description:	Tests at 25 °C, pH 7, synthetic substrate and full-scale sample
Test No.:	1 of 6
Duration	4,0 h (240 min)
Substrate:	Synthetic: Acetate (350 mg L^{-1}) + mineral solution with N and P
Sampling point:	Middle mixed liquor height in the SBR
Samples No.:	OHO.AER.1(1-22)
Total sample volume:	222 mL (10 mL for MLVSS, 6 mL for other samples)
Reactor volume:	2.5 L

Experimental procedure in short:	**Time (h:min)**
1. Confirm the availability of sampling material and required equipment.	08:00
2. Confirm calibration and functionality of systems, meters and sensors.	08:10
3. Transfer 1.25 L of sludge to batch reactor.	08:30
4. Start with gentle mixing and air sparging at T and pH set points.	08:40
5. 20 min before starting take sample for initial conditions (OHO.AER.1.1).	09:40
7. Start the test: add 1.25 L synthetic influent (minute zero).	10:00
8. Minute 5, continue sampling program according to schedule.	10:05
9. Minute 240, stop aeration and mixing.	14:00
10. Organize the samples and clean the system.	14:15
11. Verify that all systems are swtiched off.	14:20

Sampling schedule

Time (min)	-20	0	5	10	15	20	25	30	40	50	60	75
Time (h)	-0.33	0.00	0.08	0.17	0.25	0.33	0.42	0.50	0.67	0.83	1.00	1.25
Sample No.	1		2	3	4	5	6	7	8	9	10	11
Parameter	AEROBIC PHASE											
HAc (mg L^{-1})	56[1]		203	195.5	184	173	158.5	143.5	135.5	129	119.5	83
PO_4-P (mg L^{-1})	1.2[1]											
NH_4-N (mg L^{-1})	5.3[1]											
MLSS and MLVSS (mg L^{-1})	See table											See table

[1] Average value of the concentration present in the synthetic substrate and and liquid phase of the sludge sample prior to the start of the test.

Sampling schedule (continued)

Time (min)	90	105	120	135	150	165	180	195	210	225	240
Time (h)	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75	4.00
Sample No.	12	13	14	15	16	17	18	19	20	21	22
Parameter	AEROBIC PHASE										
HAc (mg L^{-1})	73	63	43	38	28	30.5	33	25.5	28	26.5	30
PO_4-P (mg L^{-1})											1.8
NH_4-N (mg L^{-1})											8.3
MLSS and MLVSS (mg L^{-1})											See table

MLSS & MLVSS measurements

Sampling point	Cup No.	W1	W2	W3	W2-W1	W2-W3	MLSS	MLVSS	Ratio
Start aerobic phase[2]	1	0.08835	0.10741	0.08849	0.01906	0.01892	1,906	1,892	0.99
	2	0.08835	0.10759	0.09018	0.01924	0.01742	1,924	1,742	0.91
	3	0.08834	0.10683	0.08940	0.01849	0.01742	1,849	1,742	0.94
						Average	1,893	1,792	0.95
End aerobic phase	4	0.08868	0.10758	0.08934	0.01890	0.01824	1,890	1,824	0.97
	5	0.08764	0.10617	0.08874	0.01853	0.01742	1,853	1,742	0.94
	6	0.08722	0.10648	0.08973	0.01926	0.01675	1,926	1,675	0.87
						Average	1,890	1,747	0.93

[2] Sample taken before substrate addition.

Biomass composition

Sampling point	Start Aer.	End Aer.
MLSS (mg L^{-1})	1,893	1,890
MLVSS (mg L^{-1})	1,792	1,747
Ratio	0.95	0.93
Ash (mg L^{-1})	101	142

Note:

Acetate (CH_2O)	30.03	mg C-mmol $^{-1}$
Orthophosphate (PO_4^{3-}-P)	31.00	mg P-mmol $^{-1}$
Ammonium (NH_4^+-N)	14.00	mg N-mmol $^{-1}$

The presence of nitrogen (as ammonia) and phosphorus (as orthophosphate) at the beginning (of around 5.3 and 1.2 mg L^{-1} in the activated sludge, respectively) and end of the test (of 8.3 and 1.8 mg L^{-1}, accordingly) indicates that these micronutrients were not limiting. Actually, the concentrations are higher at the end of the test because the synthetic influent contained around 28 mg NH_4-N L^{-1} and 8 mg PO_4-P L^{-1}. Thus, after dilution and nutrient consumption for biomass synthesis, 8.3 mg NH_4-N L^{-1} and 1.8 mg PO_4-P L^{-1} remained at the end of the aerobic test. Should the actual nitrogen (N_{req}) and phosphorus (P_{req}) requirements be known, then they can be estimated with the following expressions:

$$N_{req} = \frac{Y_{COD} \cdot RBCOD_{removed} \cdot N_S}{f_{CV}} \quad \text{Eq. 2.5.2}$$

$$P_{req} = \frac{Y_{COD} \cdot RBCOD_{removed} \cdot P_S}{f_{CV}} \quad \text{Eq. 2.5.3}$$

Where, Y_{OHO} is the biomass growth yield (in COD-biomass COD-substrate^{-1}), $RBCOD_{removed}$ is the concentration of RBCOD removed in the test (taking into account the initial COD concentration and the potential dilution effects), N_S is the nitrogen requirement for biomass growth assumed to be around 0.10 g N g VSS^{-1}, P_S is the phosphorus requirement for biomass growth assumed to be around 0.03 g P g VSS^{-1}, f_{CV} is the COD-to-VSS ratio of the sludge, commonly assumed to be 1.42 or 1.48 g COD g VSS^{-1} (Metcalf and Eddy, 2003; Ekama and Wentzel, 2008a).

2.5.7 Additional considerations and recommendations

2.5.7.1 Simultaneous storage and microbial growth

Different studies performed in lab- and full-scale systems have documented the simultaneous occurrence of RBCOD storage and microbial growth under aerobic conditions (van Loosdrecht *et al.,* 1997; Beun *et al.,* 2000; Dircks *et al.,* 2001; Martins *et al.,* 2003; Sin *et al.,* 2005). Under such conditions and when OHOs are exposed to high substrate gradients (like those observed in plug-flow reactors or in aerobic selectors in activated sludge systems), part of the RBCOD present in the bulk liquid is transported through the cell membrane and stored as intracellular polymers like PHA for their further use for microbial growth when the substrate concentrations are low or exhausted (van Loosdrecht *et al.,* 1997; Beun *et al.,* 2000, Dircks *et al.,* 2001); the remaining RBCOD fraction that is not intracellularly stored is directly used for biomass growth (Sin *et al.,* 2005). Such a combination of processes will lead to apparently higher Y_{OHO} values since the aerobic RBCOD storage processes apparently require less oxygen than the direct growth on RBCOD (at least when the substrate storage process takes place). The role and importance of storage processes in activated sludge systems has been significantly recognized to such a degree that different models have been developed and discussed to provide a better description of the simultaneous occurrence of storage and growth in full-scale systems (Gujer *et al.,* 1999; Sin *et al.,* 2005; Guisasola *et al.,* 2005, van Loosdrecht *et al.,* 2015). For the execution of aerobic matter removal tests focused on the determination of the kinetic removal rates, the simultaneous occurrence of these processes should not have direct practical implications. However, the reader should be aware that they will influence the oxygen uptake rate profiles and lead to certain deviations in the suggested Y_{OHO} values as thoroughly described elsewhere (Gujer *et al.,* 1999).

2.5.7.2 Lack of nutrients

Though it may seem trivial, the presence of macro- and micro-nutrients in the right concentrations is essential for successful operation of an activated sludge system. These are usually present in sufficient amounts in most municipal wastewaters, but their presence should be checked and confirmed particularly if the sewage plant is regularly receiving industrial effluents. For this purpose, a simple estimation of the nutrient requirements of the biomass as a function of the COD removal concentration (as suggested in this chapter) can be performed and compared with the influent nutrient concentrations to assess whether there are sufficient nutrients available to cover the biological growth requirements. Lack of such nutrients can lead to severe problems including pin floc and filamentous bulking sludge (Eikelboom, 2000; Martins, 2004), affecting the effluent quality and, in extreme cases, can lead to the failure of the activated sludge system.

2.5.7.3 Toxicity or inhibition

The abundance of OHOs in activated sludge systems is so broad and diverse that they can acclimatise and adapt to different environmental and operating conditions and even stand the presence of different potentially toxic or inhibitory compounds, particularly proceeding from industrial activities. Although they can acclimatise and adapt to such diverse conditions, the inhibitory effects can lead to sub-optimal microbial process activity. To

assess such potential effects, two batch activity tests can be executed: one with the original sludge and another one with the same sludge but washed in a mineral solution to remove potentially inhibiting or toxic compounds (as explained in Section 2.5.3.5). To compare the results obtained from each batch activity test, it will be very useful to assess whether the presence of certain compounds inhibits the activity of OHOs. However, one should be aware that such an approach may only be successful if the inhibiting effects are (rapidly) reversible.

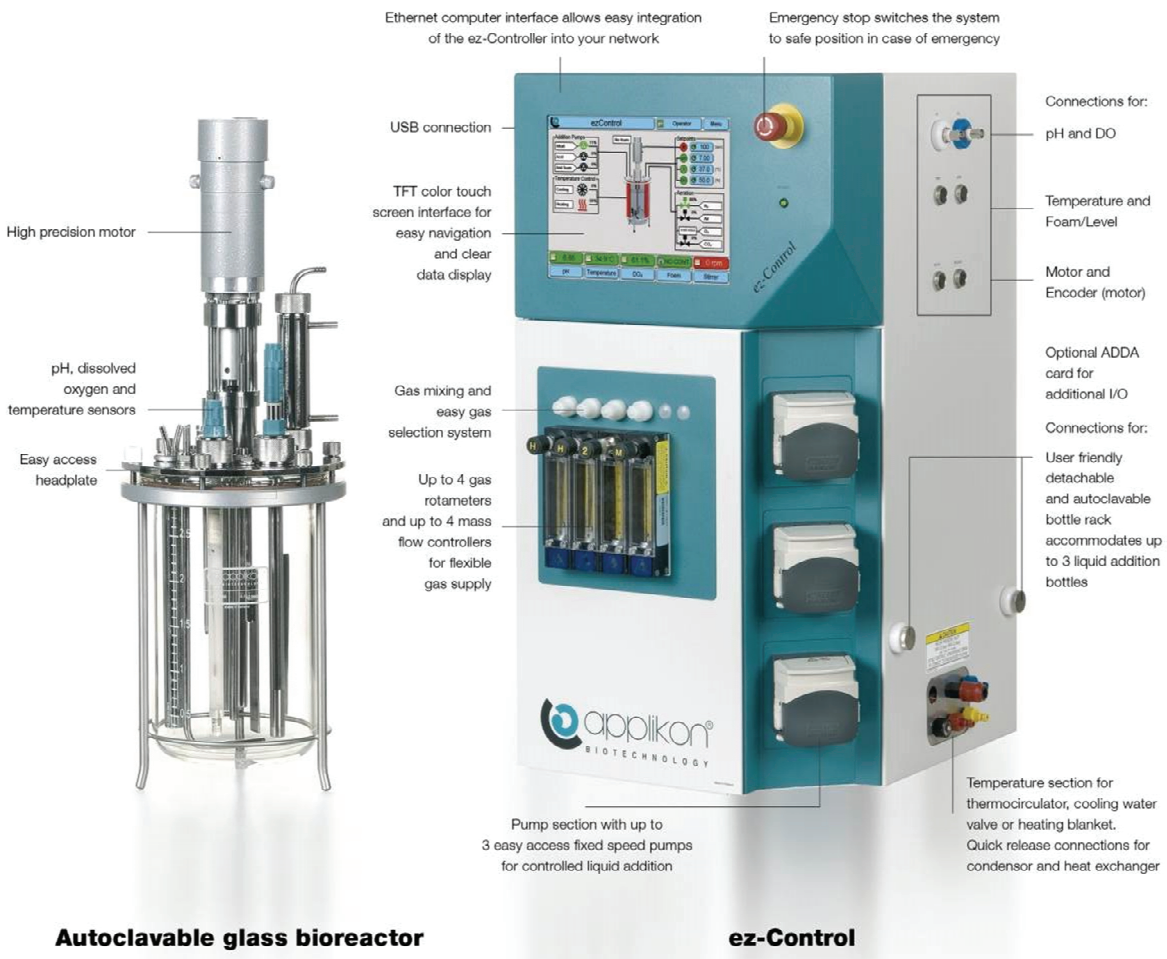

Figure 2.5.3 A high-tech bioreactor system for batch and continuous tests with microbial cultures (photo: Applikon Biotechnology B.V., 2016)

Annex I: Unit conversion coefficients

Table A1 Conversion factors (CF) for net-conversions, stoichiometric and kinetic parameters for carbon, phosphorus, nitrogen and sulphur.

Parameter	Unit (mg basis)	Unit (mole basis)	CF	Unit (COD basis)	CF
ANAEROBIC PARAMETERS					
Phosphate released/HAc uptake ratio	mg PO_4 mg $C_2H_4O_2^{-1}$	P-mol C-mol^{-1}	0.32	mg PO_4 mg COD^{-1}	0.95
Glycogen utilization/HAc uptake ratio	mg $(C_6H_{10}O_5)_n$ mg $C_2H_4O_2^{-1}$	C-mol C-mol^{-1}	1.11	mg COD mg COD^{-1}	1.12
PHB formation/HAc uptake ratio	mg $(C_4H_6O_2)_n$ mg $C_2H_4O_2^{-1}$	C-mol C-mol^{-1}	1.40	mg COD mg COD^{-1}	1.57
PHV formation/HAc uptake ratio	mg $(C_5H_8O_2)_n$ mg $C_2H_4O_2^{-1}$	C-mol C-mol^{-1}	1.50	mg COD mg COD^{-1}	1.82
PH_2MV formation/HAc uptake ratio	mg $(C_6H_{10}O_2)_n$ mg $C_3H_6O_2^{-1}$	C-mol C-mol^{-1}	1.58	mg COD mg COD^{-1}	2.01
Phosphate release/HPr uptake ratio	mg PO_4 mg $C_3H_6O_2^{-1}$	P-mol C-mol^{-1}	0.80	mg PO_4 mg COD^{-1}	0.65
Glycogen utilization/HPr uptake ratio	mg $(C_6H_{10}O_5)_n$ mg $C_3H_6O_2^{-1}$	C-mol C-mol^{-1}	0.92	mg COD mg COD^{-1}	0.77
PHB formation/HPr uptake ratio	mg $(C_4H_6O_2)_n$ mg $C_3H_6O_2^{-1}$	C-mol C-mol^{-1}	1.15	mg COD mg COD^{-1}	1.08
PHV formation/HPr uptake ratio	mg $(C_5H_8O_2)_n$ mg $C_3H_6O_2^{-1}$	C-mol C-mol^{-1}	1.23	mg COD mg COD^{-1}	1.25
PH_2MV formation/HPr uptake ratio	mg $(C_6H_{10}O_2)_n$ mg $C_3H_6O_2^{-1}$	C-mol C-mol^{-1}	1.30	mg COD mg COD^{-1}	1.39
PHV formation/PHB formation	mg $(C_5H_8O_2)_n$ mg $(C_4H_6O_2)_n^{-1}$	C-mol C-mol^{-1}	1.08	mg COD mg COD^{-1}	1.16.
Sulphate reduction/HAc uptake ratio	mg SO_4 mg $C_2H_4O_2^{-1}$	S-mol C-mol^{-1}	0.31	mg SO_4 mg COD^{-1}	0.95
Sulphate reduction/HPr uptake ratio	mg SO_4 mg $C_3H_6O_2^{-1}$	S-mol C-mol^{-1}	0.26	mg SO_4 mg COD^{-1}	0.65
Active biomass formation/Sulphate reduction	mg $CH_{2.09}O_{0.54}N_{0.20}P_{0.02}$ mg SO_4^{-1}	C-mol S-mol^{-1}	3.70	mg COD mg SO_4^{-1}	1.52
ANOXIC PARAMETERS					
PHB degradation/Nitrate removal	mg $(C_4H_6O_2)_n$ mg NO_3^{-1}	C-mol N-mol^{-1}	2.88	mg COD mg NO_3^{-1}	1.66
PHV degradation/Nitrate removal	mg $(C_5H_8O_2)_n$ mg NO_3^{-1}	C-mol N-mol^{-1}	3.10	mg COD mg NO_3^{-1}	1.92
PH_2MV degradation/Nitrate removal	mg $(C_6H_{10}O_2)_n$ mg NO_3^{-1}	C-mol N-mol^{-1}	3.26	mg COD mg NO_3^{-1}	2.12
Glycogen formation/Nitrate removal	mg $(C_6H_{10}O_5)_n$ mg NO_3^{-1}	C-mol N-mol^{-1}	2.30	mg COD mg NO_3^{-1}	1.18
Poly-P formation/Nitrate removal	mg $(PO_3Mg_{0.33}K_{0.33})_n$ mg NO_3^{-1}	P-mol N-mol^{-1}	0.62	-	-
Active biomass formation/Nitrate removal	mg $(CH_{2.09}O_{0.54}N_{0.20}P_{0.02})$ mg NO_3	C-mol N-mol^{-1}	2.38	mg COD mg NO_3^{-1}	1.52
Methanol degradation/Nitrate removal	mg CH_4O mg NO_3^{-1}	C-mol N-mol^{-1}	1.92	mg COD mg NO_3^{-1}	1.98
Ethanol degradation/Nitrate removal	mg C_2H_6O mg NO_3^{-1}	C-mol N-mol^{-1}	2.70	mg COD mg NO_3^{-1}	2.06
Acetate degradation/Nitrate removal	mg $C_2H_4O_2$ mg NO_3^{-1}	C-mol N-mol^{-1}	2.08	mg COD mg NO_3^{-1}	1.06
PHB degradation/Nitrite removal	mg $(C_4H_6O_2)_n$ mg NO_2^{-1}	C-mol N-mol^{-1}	2.14	mg COD mg NO_2^{-1}	1.66
PHV degradation/Nitrite removal	mg $(C_5H_8O_2)_n$ mg NO_2^{-1}	C-mol N-mol^{-1}	2.30	mg COD mg NO_2^{-1}	1.92
Anoxic PH_2MV degradation/Nitrite removal	mg $(C_6H_{10}O_2)_n$ mg NO_2^{-1}	C-mol N-mol^{-1}	2.42	mg COD mg NO_2^{-1}	2.12
Glycogen formation/Nitrite removal	mg $(C_6H_{10}O_5)_n$ mg NO_2^{-1}	C-mol N-mol^{-1}	1.71	mg COD mg NO_2^{-1}	1.18
Poly-P formation/Nitrite removal	mg $(PO_3Mg_{0.33}K_{0.33})_n$ mg NO_2^{-1}	P-mol N-mol^{-1}	0.46	-	-
Active biomass formation/Nitrite removal	mg $CH_{2.09}O_{0.54}N_{0.20}P_{0.02}$ mg NO_2^{-1}	C-mol N-mol^{-1}	1.77	mg COD mg NO_2^{-1}	1.52
Methanol degradation/Nitrite removal	mg CH_4O mg NO_2^{-1}	C-mol N-mol^{-1}	1.43	mg COD mg NO_2^{-1}	1.98
Ethanol degradation/Nitrite removal	mg C_2H_6O mg NO_2^{-1}	C-mol N-mol^{-1}	2.00	mg COD mg NO_2^{-1}	2.06
Acetate degradation/Nitrite removal	mg $C_2H_4O_2$ mg NO_2^{-1}	C-mol N-mol^{-1}	1.54	mg COD mg NO_2^{-1}	1.06
Nitrite removal/Ammonium removal	mg NO_2 mg NH_4^{-1}	N-mol N-mol^{-1}	0.39	mg NO_2 mg NH_4^{-1}	-
Nitrate removal/Ammonium removal	mg NO_3/mg NH_4^{-1}	N-mol N-mol^{-1}	0.29	mg NO_3 mg NH_4^{-1}	-
Act. biom. formation/Ammonium cons.	mg$(CH_{2.09}O_{0.54}N_{0.20}P_{0.02})$ mg NH_4^{-1}	C-mol N-mol^{-1}	0.69	mg COD mg NH_4^{-1}	-
AEROBIC PARAMETERS					
PHB degradation/Oxygen consumption	mg $(C_4H_6O_2)_n$ mg O_2^{-1}	C-mol mol O_2^{-1}	0.37	mg COD mg O_2^{-1}	1.66
PHV degradation/Oxygen consumption	mg $(C_5H_8O_2)_n$ mg O_2^{-1}	C-mol mol O_2^{-1}	0.40	mg COD mg O_2^{-1}	1.92
PH_2MV degradation/Oxygen consumption	mg $(C_6H_{10}O_2)_n$ mg O_2^{-1}	C-mol mol O_2^{-1}	0.42	mg COD mg O_2^{-1}	2.12
Glycogen formation/Oxygen consumption	mg $(C_6H_{10}O_5)_n$ mg O_2^{-1}	C-mol mol O_2^{-1}	0.29	mg COD mg O_2^{-1}	1.18
Poly-P formation/Oxygen consumption	mg $(PO_3Mg_{0.33}K_{0.33})_n$ mg O_2^{-1}	P-mol mol O_2^{-1}	0.08	mg $(PO_3Mg_{0.33}K_{0.33})_n$ mg O_2^{-1}	-
Active biom. formation/Oxygen cons.	mg $(CH_{2.09}O_{0.54}N_{0.20}P_{0.02})$ mg O_2^{-1}	C-mol mol O_2^{-1}	0.31	mg COD mg O_2^{-1}	1.52
Active biomass formation/Ammonium cons.	mg $(CH_{2.09}O_{0.54}N_{0.20}P_{0.02})$ mg NH_4^{-1}	C-mol N-mol^{-1}	0.69	mg COD mg NH_4^{-1}	1.52

GENERAL PARAMETERS					
NA	mg P mg C^{-1}	P-mol C-mol^{-1}	0.39	-	-
NA	mg C mg C^{-1}	C-mol C-mol^{-1}	1.00	-	-
NA	mg C mg N^{-1}	C-mol N-mol^{-1}	1.16	-	-
NA	mg P mg N^{-1}	P-mol N-mol^{-1}	0.45	-	-
NA	mg C mg O_2^{-1}	C-mol O_2-mol^{-1}	1.33	-	-
NA	mg P mg O_2^{-1}	P-mol O_2-mol^{-1}	1.04	-	-
MOLECULAR WEIGHTS					
HF	mg CH_2O_2	C-mol	0.022	mg COD	0.35
HAc	mg $C_2H_4O_2$	C-mol	0.033	mg COD	1.06
HPr	mg $C_3H_6O_2$	C-mol	0.041	mg COD	1.53
HBr	mg $C_4H_8O_2$	C-mol	0.045	mg COD	1.80
Lactate	mg $C_3H_6O_3$	C-mol	0.033	mg COD	1.06
Methanol	mg CH_4O	C-mol	0.031	mg COD	1.98
Ethanol	mg C_2H_6O	C-mol	0.043	mg COD	2.06
Glucose	mg $C_6H_{12}O_6$	C-mol	0.033	mg COD	1.06
Sulphide	mg S^{2-}	S-mol	0.031	mg COD	2.00
Carbondioxide	mg CO_2	C-mol	0.023	-	-
Phosphate	mg PO_4	P-mol	0.011	-	-
Nitrogen	mg N_2	N-mol	0.071	-	-
Ammonium	mg NH_4	N-mol	0.055	-	-
Nitrate	mg NO_3	N-mol	0.048	-	-
Nitrite	mg NO_2	N-mol	0.065	-	-
Oxygen	mg O_2	mol O_2	0.031	-	-
Sulphate	mg SO_4	S-mol	0.010	-	-
PHB	mg $(C_4H_6O_2)_n$	C-mol	0.046	mg COD	1.66
PHV	mg $(C_5H_8O_2)_n$	C-mol	0.050	mg COD	1.92
PH2MV	mg $(C_6H_{10}O_2)_n$	C-mol	0.053	mg COD	2.12
Glycogen	mg $(C_6H_{10}O_5)_n$	C-mol	0.037	mg COD	1.18
Poly-P	mg $(PO_3Mg_{0.33}K_{0.33})_n$	P-mol	0.010	-	-
Active biomass	mg $(CH_{2.09}O_{0.54}N_{0.20}P_{0.02})$	C-mol	0.038	mg COD	1.52
ADDITIONAL FACTORS					
Common active biomass fraction of VSS in EBPR enrichment cultures [a]	mg VSS	mg active biomass	0.80		-

[a] Glycogen content of the sludge varies, dependent on the poly-P content (Welles *et al.,* 2015b).

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