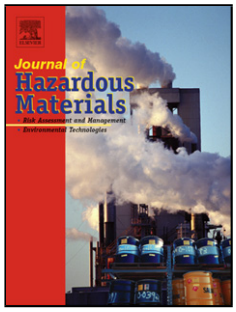

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Toxicity Measurement in Biological Wastewater Treatment Processes: a Review

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Highlights

- Different methods for toxicity assessment in WW were critically reviewed.
- Laboratory standardization of the anaerobic ATA assay is urgently needed
- Biosensor matrix or a biosensor with multiple reporter organisms needed for real WW
- MFC-based biosensors need correlation with traditional assays in real WWs
- On-line data processing methods need to be developed to interpret data from sensors

Abstract

Biological wastewater treatment processes (WWTPs), by nature of their reliance on biological entities to degrade organics and sometimes remove nutrients, are vulnerable to toxicants present in their influent. Various toxicity measurement methods have been adopted for biological WWTPs, but most are performed off-line, and cannot be adapted to on-line monitoring tools to provide an early warning for WWTP operators. However, the past decade has seen a rapid expansion in the research and development of biosensors that can be used for toxicity assessment of aquatic environments. Some of these biosensors have also been shown

to be effective for use in biological WWTPs. Nevertheless, more research is needed to: examine the sensitivity of assays and sensors based on single organisms to various toxicants and develop a matrix of biosensors or a biosensor incorporating multiple organisms that can protect WWTPs; test the micro fuel cell (MFC)-based biosensors with real wastewaters and correlate the results with the well-established oxygen uptake rate (OUR)-based or CH_4-based toxicity assay; and, develop advanced data processing methods for interpreting the results of on-line toxicity sensors in real WWTPs to reduce the noise due to the normal fluctuation in influent quality and quantity.

Key words: biological wastewater treatment, activated sludge, anaerobic digestion, toxicity, biosensor

1. Introduction

Biological WWTP has been used for more than 100 years and to date it continues to maintain a prominent presence within the environmental protection infrastructure. Sustaining a high level of biological activity is critical to the operation of a biological WWTP, however, being a biological system it is inherently susceptible to the debilitating effects of toxic compounds in its influent, which often leads to disruption in operation within WWTPs. A recent report documented that a severe cyanide spill caused the upset of a WWTP in UK and the death of thousands of fish in the river receiving the effluent [1]; in another case, an illegal chemical dumping incident caused the shutdown of a WWTP [2]. The importance of an early warning system (EWS) that can detect toxicity in WWTPs was also highlighted in a survey done by the Water Environment Research Foundation (WERF, Alexandria, VA): an EWS was widely (>83%) believed to be important, but much less (<50%) implemented in the wastewater treatment industry [3]. Due to the complexity of wastewater, it is often difficult to

identify the specific toxicants that result in the declining performance of a particular WWTP [4], and therefore, prompt measurement of toxicity is vital to the smooth operation of WWTPs.

Among the diverse biological wastewater treatment methods, aerobic processes, especially activated sludge, dominates. Thus, most of the new toxicity assays that have been developed focus on their application to activated sludge. Recent advances in membrane technology have made anaerobic wastewater treatment a very attractive process [5]. However, anaerobic processes, especially their methanogenic components, are very sensitive to toxic compounds [6]. Well-known toxic compounds including ammonia, sulfide, volatile fatty acids, metal ions and organics, can disrupt the anaerobic digestion processes to varying degrees. Despite the general recognition that prompt detection of toxic compounds is vital to the smooth operation of anaerobic processes, there has been little advance in measuring toxicity to anaerobic process in the past three decades. Meanwhile, research and development within the domain of biosensors has bloomed [7–10], and some of these biosensors clearly have the potential to be used in the context of biological WWTPs. This review will cover toxicity measurement methods for both aerobic and anaerobic processes, and present a range of promising biosensors that may be adopted for use in WWTPs. Although ecotoxicological assays – such as those based on the growth inhibition of algae or plants, the mortality of crustaceans, and the mobility inhibition of *Daphnia magna* – are sometimes employed in the assessment of toxicity to wastewater treatment processes [11], they are fundamentally designed to reflect the ecological impact of toxic compounds to aquatic environments [12]. This is distinct from the need for acute toxicity alerts relevant to WWTPs, and hence these assays have not been included in the scope of this review.

2. Approaches to toxicity measurement

Toxicity is the extent to which a toxic compound (toxicant) can harm an organism or a group of organisms. The extent of damage can be measured directly by the deterioration of physiological functions of the organisms, such as oxygen uptake rate (OUR) [13,14], methane production rate [15], floc size distributions [16] and community changes [17,18]. In the context of wastewater treatment, toxicity can also be measured indirectly by assessing the changes in water quality parameters, e.g. chemical oxygen demand (COD) or biochemical oxygen demand (BOD).

Rozzi and Remigi [19] provided a good review on the approaches which could be used for the measurement of activity in anaerobic processes. As it is a process with complex biochemical reactions, the approaches to measuring these toxic effects can be divided into three categories; reactant consumption, indicator of organism properties, and product generation, as shown in Table 1. The most common reactant in the aerobic process is dissolved oxygen, thus the OUR is the most widely used measurement of toxicity for aerobic processes. CO_2 production in aerobic processes has also been used for toxicity assessment, although not as widely. OUR and CO_2 production, being specific to aerobic processes, are termed respirometric methods. With more biological nutrient removal processes being used in WWTPs, toxicity to nitrification has attracted considerable research effort in the past years. Hence, changes in ammonium consumption, nitrite consumption/accumulation and nitrate accumulation have all been used in toxicity assays for the nitrification process. For anaerobic process, the easiest measurement that can be made to assess toxicity is CH_4 production, while volatile fatty acid (VFA) accumulation has also been used. The activity or toxicity of the processes can also be determined by some indicator parameters of organisms inherent to the system, i.e. the indigenous microbial community of the wastewater, such as their cell density

(growth), cell viability, floc morphology, and enzymatic activity including adenosine triphosphate (ATP) and dehydrogenase.

In addition to the assays with activated sludge or anaerobic sludge as the target, toxicity can also be estimated using indicator organisms. This is considered an indirect measurement because the indicator organisms are exogenous to the wastewater community. The bioluminescence assay is the most well-known of such methods, and recently biosensors based on enzymes or whole bacterial cells have been studied extensively. Sometimes a biosensor based on a large scale full performance reactor has also been used [20]: using this type of biosensor is a fairly good mimic of a direct measurement, but there will always be some difference between the microbial community in reactors and the one in the biosensors. For this reason, they will still be considered as indirect methods in this paper. In the following two sections, we shall further examine each assay in greater detail.

3. Direct toxicity measurement methods

3.1. OUR based respiration inhibition test

OUR based respiration inhibition is one of the oldest methods of measuring the toxicity of influent to activated sludge, and has been established and standardized over the past three decades, and further updated recently [21,22]. The method involves incubating the activated sludge with toxic compounds in a sealed container for 3 hours (or 30 minutes for volatile or hydrolytic compounds), and then monitoring the dissolved oxygen for 10 minutes with an oxygen electrode to determine the OUR. The inhibition, defined as $\%I = 1\text{-}OUR_{test}/OUR_{ctrl}$, in which OUR_{test} is the OUR of the test sample and OUR_{ctrl} is the OUR of the control sample, is then calculated [22]. In order to ensure that the biomass is sensitive and the test works properly, a reference toxicant, 3,5-dichlorophenol (3,5-DCP) is recommended to be include in the test. The medium used for the test is a synthetic sewage, which contains peptone, meat extract and urea. The OUR-based respiration inhibition test has been widely used for the toxicity assessment of organics [23,24], heavy metals: chromium (VI) [25] and Ni(II), Cr

(III) and Zn (II) [26], and complex wastewater, e.g., landfill leachate [27], to activated sludge. As a standard method, it is widely used for comparison with other new methods [28,29].

One of the factors that prevents the OUR based respiration inhibition test from being used as an early warning assay is its long response time of 3 hours; thus some modifications to the method were proposed recently to decrease the exposure time. Tzoris et al. [29,30] developed a portable manometric respirometer, which measured the oxygen usage via a pressure transducer in a small cell (4.3 mL). The response time of the device can be as low as 6 minutes.

The OUR-based respiration inhibition test itself does not differentiate between heterotrophic and autotrophic oxidation (nitrification). However, if this distinction is required, an inhibitor of nitrification, e.g., N-allythiourea (ATU) can be added to the test, which will give OUR results based only on heterotrophic oxidation [22].

3.2. Carbon dioxide evolution test

The carbon dioxide evolution test, also known as the modified Sturm test, has (similarly to the OUR method) been established and continuously updated for almost 3 decades [31]. The test was designed for evaluating the biodegradability of non-volatile compounds in liquid medium. The standard CO_2 evolution test involves aerating a measured volume of sample and inoculum with CO_2-free air for 28 days; the CO_2 produced was trapped in alkaline solution and measured by titration. When this method is used as a toxicity assay, an easily-biodegradable substrate is also added, and the ratio of CO_2 production with a toxicant in it to one without a toxicant can be used as an indication of the degree of toxicity. Compared to an OUR test, the CO_2 evolution test features longer incubation times, and thus is more suitable as a biodegradability and chronic toxicity assay.

In real applications, different modifications to the standard method have been used, including shorter incubation times and no aeration. For example, Vermuë et al. [32] used 7-

day CO_2 production to measure the toxicities of 58 water-immiscible organic solvents to several test microorganisms; the headspace samples were analyzed after 1 day and 7 days of incubation with a GC. The CO_2 production after 7 days in samples with toxic compounds relative to the ones without was defined as the inhibition. Recently, Strotmann et al. [33] developed an online CO_2 evolution test, which traps CO_2 in KOH or NaOH solutions and measures conductivity continuously. The change in conductivity correlates well with the CO_2 production.

The CO_2 evolution test has been used in the ecotoxicological assessment of drilling fluids [34] and oilfield wastewater [35]. One of the few example where it was used for assessing the toxicity in wastewater treatment processes was by Prado et al. [36], who used the CO_2 evolution test to evaluate the toxicity of veterinary antibiotics tetracycline and tylosine to activated sludge. The tests were performed in 1-L batch reactors and sodium benzoate was also used as the reference compound.

3.3. Nitrification/denitrification inhibition assay

3.3.1. Ammonium/Nitrite/Nitrate based nitrification/denitrification inhibition assay

Due to its significant sensitivity to toxicants [37], toxicity assays specific to nitrification have been developed. For example, in addition to the OUR-based nitrification inhibition test mentioned in the previous section, an ammonium/nitrite-based nitrification inhibition assay was developed [38]. The assay employed two test microorganisms: an ammonia-oxidizing *Nitrosomonas* sp. and a nitrite-oxidizing *Nitrobacter*, sp., which were incubated in a test tube with samples for 4 hours. The ammonium and nitrite consumption were determined colorimetrically, and the consumption rates relative to the controls determined inhibition; the method has been standardized recently [39].

Odokuma and Akponah [34] used the ammonia/nitrite-based nitrification inhibition assay to assess the toxicity of four drilling fluids; for both tests nitrite accumulation or

consumption was monitored. The CO_2 evolution test using *Escherichia coli* as the indicator organism was also included for comparison. They concluded that the nitrite oxidization process is more sensitive to toxicants than the CO_2-evolution test with *E. coli*, and thus could give a quicker response (within 8 hours). The nitrite production-based nitrification inhibition assay has been applied to evaluate the toxicity of silver nanoparticles (Ag-NPs) to ammonia oxidizing bacterium (AOB) *Nitrosomonas europaea* [40], leading to the distinction of level of toxicity between small Ag-NPs (20 nm) and large Ag-NPs (80 nm). The ammonia consumption-based nitrification inhibition test, on the other hand, has been successfully applied to monitor the toxicity of 2,4-dinitroanisole (DNAN), a munition compound [41], naphthenic acid [42], antibiotics colistin sulfate [43] and tetracycline [44], and S^{2-} [45].

Like the nitrification inhibition assay, a denitrification inhibition assay based on the consumption of nitrate can be used to evaluate the toxicity, for example, of Cu(II) [46]. The test was able to discern that denitrifying bacteria are the most sensitive to Cu(II) among the trophic groups assessed (the other groups being fermentative bacteria, aerobic heterotrophs, and nitrifying bacteria).

3.3.2. Nitrous oxide accumulation based nitrification inhibition assay

An increase in nitrous oxide (N_2O) in the off-gas of wastewater treatment processes was found to correlate well with ammonia shock loads and oxygen depletion to nitrification process, and hence it is useful for monitoring nitrification [47], and as a component of an early warning system [48].

3.4. Methane production-based anaerobic toxicity assay

The CH_4 production based anaerobic toxicity assay (ATA) described by Owen and colleagues [15,49] is the most widely used method for evaluating the toxicity of substrates to methanogenesis. The assay was initially developed by Nottingham and Hungate [50] and Miller and Wolin [51]. Similar to the CO_2 evolution test, ATA measures biogas production in

a batch set-up and uses the gas production ratio to the controls to evaluate the toxicity of the substrates. The method is very convenient and cheap to employ, and has been applied in toxicity testing of a wide variety of substrates including plasticizers, halogenated phenols [52–54], nitroaromatics and metals [55].

To use the ATA an accurate measurement of biogas volume is critical. The commonly used methods include volume displacement devices, e.g., syringes and manometers, and pressure transducers. When pressure transducers are used, biogas volume is measured from the increased pressure inside the reactors using a pressure transducer. Since the biogas produced is not released in the closed system and could easily dissolve in the aqueous phase under high pressure, errors could be induced in this method when the calibration does not include this error. When manometers are used, the trapping solution should be chosen carefully as Walker et al. [56] showed that different trapping solutions could generate systematic errors (which are often ignored by researchers). As suggested by the group, an acidified (pH 2) 75% saturated NaCl solution was the best barrier solution. Among all the methods, lubricated syringes are still the most widely used due to their ease of use and accuracy. Some automated biogas volume measurement devices have also been developed [57] and commercialized [58].

A combination of volatile fatty acids (VFAs) including acetic acid, propionic acid and butyric acid are used most of the time as the substrate for ATAs, although sometimes the sole use of acetic acid, glucose or ethanol may be required. When VFAs are used, the assay can only show the toxic effects on the acetoclastic methanogens. When needed, a phase-separated ATA can be conducted. For example, Shin et al. [59] devised a phase-separated ATA to study the toxicity of sulfate and sulfide to both the acetoclastic methanogens and the acidogenic anaerobes. The substrate used for the acidogenic ATA was glucose, while acetic acid was used for methanogens.

In applications, different modifications have been incorporated by researchers based on specific needs and contexts. In one modified ATA [60], methanol was used to dissolve toxic compounds; when gas production from methanol ceased, a spike of acetic acid/propionic acid was injected into the serum bottles, after which a standard ATA was performed. In another case [61], propylene glycol instead of acetic acid was used as the major substrate for the toxicity assay of methylbenzotriazole in aircraft deicing fluid. However, different substrates will have significant effects on the toxicity; Liu and Hegemann [62] observed that when incubated with glucose, the IC_{50} (the concentration causing 50% inhibition) of formaldehyde was 300 mg/L, but this decreased to 150 mg/L when incubated with real wood-glue wastewater. This suggests that in real wastewater the toxicity determined from simple substrates may be biased.

Most of the ATAs were done in batch reactors, however, a semi-continuous bioassay can also be used [49]. In the semi-continuous bioassay, a spike of toxic compounds and some fresh sludge were added daily to the assay. As the authors concluded, semi-continuous assays are slow and expensive, however, they provide information on the toxicity of chemicals fed continuously, and this would be encountered more frequently in real situations than one that is mimicked by a batch reactor. To interpret the results of an ATA, one of the commonly used method [63–70] is to determine the specific methanogenic activity (SMA) of the samples with toxicants supplemented (SMA_{test}) and the SMA of controls (SMA_{ctrl}), and to calculate the %IC, which is defined as $\%IC = 1\text{-}SMA_{test}/SMA_{ctrl}$. Another widely used method [71–76] is based on the total volumes of gas production of test samples (V_{test}) and controls (V_{ctrl}) within certain days, in which case, $\%IC = 1\text{-}V_{test}/V_{ctrl}$.

Most of the ATAs were conducted on mesophilic anaerobic digestion sludge (at ~ 35 °C). A few tests performed under thermophilic conditions [73,77] have shown that thermophilic anaerobic digestion sludge could be slightly less sensitive than mesophilic

sludge to toxicants. The major disadvantage of the ATA is that it is time-consuming, and sometimes the test may last for a week. Thus, it cannot be used as a prompt measurement in industry to provide alerts about toxic influents in WWTP operations. Another challenge to apply the method is that it is difficult to compare the toxicity results tested in different labs, as no standardized conditions have been applied across the board.

3.5. Enzymatic activity of microorganisms

3.5.1. ATP luminescence assay

As the most important energy "currency" in living systems, ATP concentrations are directly affected by the physiological conditions of organisms. The ATP luminescence assay is based on measuring the decrease in ATP content in organisms when they are exposed to toxicants. ATP is quantified through the activity of firefly luciferase, which converts ATP to AMP and emits light. Its use on activated sludge has been established for a very long time [78]. The assay involves about 15-40 minutes of ATP extraction, before determination of ATP levels through the luminescence produced by luciferase [79,80]. Dalzell et al. [28] compared the ATP luminescence assay with the OUR-based respiration inhibition test and nitrification inhibition assay for toxicity assessment of activated sludge. They found that among the three methods, the ATP luminescence assay was the least sensitive to toluene, Cd, Zn, and the most sensitive to Cu; the nitrification inhibition assay was the most sensitive assay overall. This assay has been included to complement the biodegradability test which is used to assess the toxicity of imidazolium ionic liquids [81].

3.5.2. Dehydrogenase activity

The dehydrogenase enzyme is one of a major group of enzymes involved in the electron transfer processes of organisms. Therefore, its activity is a good indicator of the collective metabolic activity of microbial communities such as the ones involved in

wastewater treatment. The dehydrogenase activity assay is based on the spectrophotometric measurement of electron-acceptor dyes, such as triphenyltetrazolium chloride (TTC) and resazurin, which can be reduced by the dehydrogenases quickly [82], and amongst them the assay based on resazurin is the most widely used. Commercial kits based on this assay, e.g., ToxTrak^{TM} (HACH, Loveland, CO, US) using either pure strains or mixed cultures are available [83]. With a response time of 45 minutes, the method can be adopted as a quick screening step to detect influent toxicity, however, unlike the ATP luminescence assay there is no need to extract the dehydrogenase from the cells. Thus, it can be used *in vivo* and possibly *in situ*. The dehydrogenase assay based on reduction of tetrazolium dye has further been developed into a commercial cell membrane integrity assay [84], which will be discussed in the following section.

Strotmann et al. [85] compared the resazurin dehydrogenase assay with the OUR-based respiratory inhibition assay for assessing the toxicity of different phenolic compounds to activated sludge. They found that the dehydrogenase assay was a reliable method, although the results did not always correlate well with the respiratory inhibition assay, and furthermore, that pH had an important effect on the results. However, in a recent study using pure cultures, the dehydrogenase assay was found to correlate well with the OUR assay [86]. The assay was also used for assessing the toxicity of Cu(II) [87] and imidazolium ionic liquids [24] to activated sludge. El Bestawy et al. [88] used the method (with TTC instead of resazurin) for assaying the toxicity of heavy metals (Cu, Cd, Co and Cr) to activated sludge: the results of the dehydrogenase assay corresponded well with the OUR assay.

3.6. Cell growth inhibition

For pure cultures cultivated under laboratory conditions, the change in growth of cells monitored via optical density (OD) is a simple and common way to assess toxicity. In wastewater treatment processes, however, the growth of cells within the sludge is generally

minimal in the short span of time used for toxicity assays, and changes cannot be satisfactorily measured using this technique. However, it can be used together with other methods to provide additional information. Xu et al. [89] used a turbidimetric microtiter assay on a medium-throughput platform to quantify the changes in growth (OD_{600}) of activated sludge in the presence of melamine, a common ingredient of plastics. The OD_{600} was monitored every 1 hour for 72 hours. The results showed that with melamine as the only carbon source, no increase in the OD_{600} of the activated sludge was observed. When an indicator culture is available, growth zone inhibition on agar plates was also used to assay the toxicity [90].

3.7. Mortality/viability assay

3.7.1. Mortality assay

The mortality or viability of target organisms is a measurement of toxicity. Depending on whether the organisms tested for viability are indigenous to the system exposed to the toxic compounds, or exogenous and serving only as an indicator organism, the assays may be considered direct or indirect, respectively. Lee et al. [91] studied the toxicity of silver nanoparticles to three model microorganisms – Gram-negative *E. coli*, Gram-positive *Bacillus subtilis*, and the yeast *Saccharomyces cerevisiae* – by exposing their pre-grown cultures to nanoparticles for varied times, and then spread plating on agar media. The colony forming unit (CFU) was quantified after 24 hours incubation and compared with the controls to reflect the toxicity. In another test, the decrease in CFUs after exposure to toxicants for certain time (48 hours) was an effective measurement of toxicity of drilling fluid to a pure culture *E. coli* [34]. Due to the need to count CFU, if the toxicity to a sludge community needs to be assessed, only the toxicity with respect to the agar-cultivable subpopulation can be assessed by this method. Thus, the CFU-based mortality/viability assay is more suitable when a specific group of organisms, which can be cultivated, is the target.

In an interesting study by Madoni et al. [17], the authors used the mortality of a protozoan community in activated sludge as an indicator of the toxicity of heavy metals. The cell numbers of 14 species of ciliated protozoa and 2 species of testate amoebae, before and after exposure to heavy meals for 24 hours, were counted microscopically to determine their toxicity.

3.7.2. Cell membrane integrity assay

One common way of assessing bacterial viability after exposure to toxicants is through the monitoring of their cell membrane integrity. The Live/Dead® BacLight™ bacterial viability stain is a popular kit used for this purpose, and is essentially a mixture of two fluorescent dyes: SYTO9 and propidium iodide (PI). SYTO9 stains the nucleic acids of all bacteria, but if the cell membranes are damaged, PI will also enter the cells, resulting in emission of red fluorescence [92] instead of green if only SYTO9 is present. The method has been used together with the nitrification inhibition test to evaluate the toxicity of the antibiotic erythromycin to activated sludge [93]. Observation under confocal laser scanning microscope (CLSM) allowed the Live/Dead® stained cells to be visualized within the flocs, and filamentous bacteria were found to be the least sensitive group to erythromycin. In another study, the Live/Dead® staining kit was used to assess the toxicity of long-chain fatty acids (LCFA): oleate, stearate and palmitate, to pure methanogenic cultures [94]. *Methanospirillum hungatei* was found to be more sensitive than *Methanobacterium formicicum* to oleate, with 99±1% and 53±10% of damaged cells, respectively, at the concentrations tested.

Cell membrane integrity can also be determined by measuring the release of intracellular K^+. Radniecki et al. [95] used this method together with the nitrite production-based nitrification inhibition assay to measure the toxicity of Ag-NPs. After the AOB *N. europaea* was exposed to Ag-NPs for 3 hours, the cells were digested in nitric acid for 24

hours and the K^+ was determined via inductively coupled plasma emission spectrometry (ICP-ES). When the toxicant concentrations increased (Ag^+: 0 – 1 ppm and 20 nm Ag-NPs: 0-10 ppm), the intracellular K^+ decreased significantly, which suggested toxicity affected outer-membrane integrity. The same method was used on activated sludge [96]. They measured significant potassium (K^+) efflux from activated sludge flocs to the bulk phase when the sludge encountered some toxic electrophilic chemicals and the flocs disintegrated; the response time was short (within minutes).

The lactate dehydrogenase (LDH) assay is another measurement of cell membrane integrity: when cells are damaged, LDH, one of the key enzymes involved in metabolism in both prokaryotes and eukaryotes, is released. The method is more often used in ecotoxicology assays [97,98] compared to toxicity assays for biological water treatment processes. One example was published very recently [99] assessing the toxicity of copper nanoparticles (Cu-NP) to activated sludge; the results showed that the increasing release of LDH correlated well with decreasing OUR and growth rate due to the increase in concentration of Cu-NP.

3.8. Floc morphology

Microscopic observation of sludge flocs has long been used to characterize sludge. Recently, with advanced image processing algorithms, it became possible to gain information on floc size, fractions of flocs with different sizes, and the fraction of filamentous bacteria [100,101]. Heine et al. [102] developed an algorithm that retrieved floc morphology information from microscopic images of activated sludge samples, and utilized the information to evaluate the condition of the biological process. They found that the filamentous fraction in the flocs was a good indicator of sludge bulking, which responded 1 or 2 days earlier than the sludge volume index (SVI), and that the microfloc fractions increased immediately after the toxicant was introduced to the system. A similar method was also used by Nisar et al. [103].

With the aid of the Live/Dead® kit, Louvet et al. [93] was able to observe the exposure time-kill activity of activated sludge samples exposed to different concentrations of erythromycin, an antibiotic. After 1 hour exposure to 0.1 mg/L and 1 mg/L erythromycin, respectively, 67% and 37% of live cells remained while 94% of live cells were left in the controls, while the floc size distribution also showed that the fraction of small flocs (<50 µm) increased significantly after being exposed to 1 mg/L erythromycin for 3 hours.

4. Indirect toxicity measurement methods

4.1. Bioluminescent assay

Bioluminescent bacteria emit luminescence through the expression of a bacterial luciferase (*lux*) gene. Bacterial luciferase catalyzes the oxidation of long-chain aliphatic aldehydes and in the process emits blue-green light [104]. The use of bioluminescent bacteria in short-term toxicity assays has been studied for more than 30 years [105]. Initially, the marine bacterium *Vibrio fischerii (Photobacterium phosphoreum),* in which bacterial bioluminescence was first observed, was used directly [106,107]. The assays featured a 'lights-off' mode, i.e. luminescence signals which should be emitted by viable *V. fischerii* cells at a certain cell density "switch off" when there are toxic compounds that affect the bacteria. Eventually, an off-line toxicity assay system for measuring the bioluminescence of *V. fischerii* (reconstituted from freeze-dried stock as part of the assay kit) was developed and marketed commercially (as Microtox®), and this has been an industrial standard method for rapid toxicity screening [108]. The Microtox® assay performs acute toxicity tests within 5 or 15 minutes [109], and can be measured not only by its proprietary Microtox® equipment, but also on microplate luminometers [110].

In environmental conditions that differ too greatly from its native ones, the marine bacteria may fail to respond biologically as expected [16]. Thus, genetically modified bacteria with a *lux* gene inserted have been constructed and used as alternative indicator

organisms [110]. In some engineered bacteria, the *lux* gene was regulated by specific promoters that respond to environmental signals and are not constitutively expressed. Thus, these bacteria feature a 'lights-on' mode, i.e, the luminescence signal increases when toxic compounds induce the promoter [104]. In such instances, the bacterium would, in effect, be serving as a whole-cell biosensor (which will be discussed in the next section) A big advantage of engineered bioluminescent bacteria is that they can be designed for specific toxic compounds or for specific effects, e.g., DNA damage, protein damage, and cell membrane damage [104]. For example, Choi and Gu [111] used a recombinant strain to successfully differentiate the chemicals with membrane-damaging effects from those with DNA-damaging or oxidative-damaging effects. In another study [112], four freeze-dried recombinant bioluminescent bacteria, which were sensitive to DNA damage, cellular membrane damage, oxidative damage and protein damage, were used to test the toxicity of endocrine disrupting chemicals (EDCs). The results showed that although there were no estrogenic effects on bacteria, some EDCs were indeed toxic to bacteria.

The bioluminescent assay has been widely used in the toxicity assessment of aquatic, air and soil environments [98,113], drinking water [104] and wastewater [106,114–117]. In the wastewater industry its focus has been on the toxicity measurement of the effluent, and on monitoring of toxicity reduction during the treatment processes [118–120]. In order to apply the bioluminescent assay to the toxicity measurement of wastewater influent, a recombinant bioluminescent bacterium *Pseudomonas fluorescens* strain Shk1, genetically modified from a strain originally isolated from activated sludge, was constructed [121]. The test results showed that the Shk1 could detect the toxicity of zinc in influent at concentrations lower than the ones detected by OUR respiration inhibition [13,16].

Although the use of a bioluminescent assay has been in place for a long time, many challenges still remain before it can be adopted as an on-line toxicity warning system. One of

the key challenges is in the maintenance of the reporter cells' viability and reactivity. Ren and Frymier tried to use a two-stage system with cell growth and cell exposure [122] units and a three-stage system with cell storage, cell growth and cell exposure [123] to resolve the issue. However, the stability of the system has to be further improved before it can be applied in industrial settings. Recently, an automated on-line bioluminescence assay system – TOXcontrol® was developed [124]. The system is totally automated except that the bioreporter cells have to be prepared in batch, which can then last for 2-5 days depending on the temperature.

Another difficulty using bioluminescent assays for wastewater processes is the sensitivity of the assay, since it is very sensitive at low concentrations of toxic compounds, e.g., < 10 nM for mitomycin C [125]. However, as an indirect measurement method, they may be too sensitive and frequently trigger false-positive signals in a biological wastewater treatment system. Lajoie et al. [13] compared bioluminescence with the OUR-based respiration inhibition assay for the toxicity of zinc in wastewater treatment. As expected, bioluminescent assays were more sensitive than the OUR assay. In another field-scale study [126], no quantitative correlation was found between the signals of a continuous bioluminescent assay and treatment performance (biological oxygen demand (BOD) removal rates). Hence, bioassays relevant to wastewater are urgently needed, and some progress has been achieved in a new bioassay developed by Marugán et al based on the use of fern spores [120] which were less sensitive than *V. fischeri* and displayed more realistic results. In addition to bacterial bioluminescence, fungal bioluminescence has recently been developed in a toxicity assay for contaminated soil samples [127], and a toxicity assay based on the algae *Scenedesmus obliquus* has been applied to wastewater samples [128].

4.2. Biosensors

A biosensor features a biological recognition element working together with a signal transducer to generate a signal proportional to the concentrations of analytes. The past two decades has seen a dramatic increase in the variety of biosensors studied and developed [10,129]. The biological recognition element could be enzymes, cofactors, antibodies, microbial cells, organelles, tissues or cells from higher organisms [10]. Amongst them, enzymes are the most widely used due to their specificity and sensitivity. However, purification of enzymes is very time-consuming and often requires stringent conditions to maintain the integrity of the purified enzymes. In comparison, whole-cell based biosensors are far easier to generate as it simply involves a cultivation of the appropriate bacterial strains, which also tend to be more robust than purified enzymes. The majority of research on enzymatic biosensors have focused on the quantitative detection of target compounds, thus, sensitivity and specificity are the two key parameters that research has primarily focused on. However, for toxicity assays, especially those that are intended for on-line measurement of toxicity in biological WWTPs, generality and reasonable sensitivity are the desired outcomes: in this respect, whole-cell biosensors are inherently more suitable. Depending on the type of transducer adopted, a biosensor may be broadly categorized as an electrochemical or an optical biosensor. Amperometric, potentiometric, conductimetric, and microbial fuel cell type biosensors are the major varieties of electrochemical biosensors [129], while bioluminescence, fluorescence, and colorimetric biosensors constitute the optical biosensor category [8]. Due to the large number of publications on biosensors, this review shall only cover studies that are relevant to biological WWTPs. For other biosensor areas readers are recommended to recent reviews [10,129–132].

4.2.1. Whole-cell biosensors

Among the whole-cell biosensors, prokaryote-based ones are the most common type [10,129]. Bacteria such as *E.coli* and *Pseudomonas putida* with the bioluminescent gene *lux* or green fluorescent protein (*gfp*) gene fused into their genome were widely explored for sensing various compounds including tetracycline, naphthalene, toluene, and methyl viologen [10]. Recently, an online flow-through bioluminescence device based on disposable biochips immobilized with genetically modified *E.coli* has been developed [133]. The luminescence signals were probed by single-photon avalanche diode detectors. Three effects-specific (DNA damage, oxidative stress, heavy metals) "light-on" promoter strains and a constitutive "light-off" promoter strain were immobilized on the biochip. In 10-days of continuous operation the device detected all the model toxicants tested: nalidixic acid, paraquat and arsenic, and an industrial wastewater sample. In another test [134], the cyanobacteria *Anabaena torulosa* trapped on a cellulose membrane was used to assess the toxicity of Cu, Pb, and Cd, 2,4-dichlorophenoxyacetate (2,4-D), and chlorpyrifos. As in the case of many bacterially based biosensors, an optical fiber connected to the fluorescence spectrometer was used as the transducer. When toxicants were present in the water, the photosynthetic activity of the cyanobacteria changed resulting in a change in fluorescence which was detected by the spectrometer. The biosensor effectively detected the toxicants at concentrations of < 10 µg/L.

Recently, whole-cell biosensors based on eukaryotes have been reported [135]; a group of protozoa known as ciliates were incorporated into biosensors. The advantage of using ciliates is that they might be more sensitive due to the lack of a cell wall, and be good alternatives to cells as animals such as ciliates and humans share more orthologs. The *gfp* gene was fused to a promoter gene, the *CdMT* gene of the ciliate: fluorescence microscopy was then used to observe the induced cells, and hence if suitable transducers are attached a viable biosensor may be generated.

When electrochemical transducers are used, major whole-cell biosensors utilize an oxygen electrode as the transducer, however, low and unstable concentrations of dissolved oxygen [136] have led to the biosensor being based on electron mediators. Wang et al [137] have developed a *p*-benzoquinone-mediated biosensor with an immobilized bacterium *Psychrobacter* sp. as the recognition element to measure the toxicity of heavy metals: Cu, Cd, Zn, Cr, Hg and Pb. When a constant potential was applied between the working and reference electrode, the change of current between the two electrodes was measured and correlated to the concentration of toxicants; the measured IC_{50} for the heavy metals varied between 1-110 mg/L. So far, most of the mediators have been added to the aqueous solution at a relative high concentration, which could cause secondary pollution, and a short life-time of the immobilized cells. Immobilization of a mediator with bacterial cells would solve this problem, and Li et al [136] successfully immobilized the mediator – benzoquinone and *E.coli* cells on a glassy carbon electrode. The biosensor could detect the toxicity of Hg, Cu, and Cd in an industrial wastewater. Zhang et al [138] developed a biosensor with the AOB *N. europaea* as the bioreceptor, and an ammonium-selective electrode as the transducer, and this sensor might be suitable in a toxicity assay for the nitrification processes.

Normally, mid-log phase cells are harvested and immobilized to make the whole-cell biosensor, which gives it a very short shelf-life. To overcome this, a whole-cell biosensor via immobilizing freeze-dried *E.coli* cells was developed [139]; the bio-sensor was reconstituted immediately prior to use with the substrates lactate, succinate and glucose. When there were toxicants in the substrate, the changes in the respiratory activity of the cells will shift the shuttle rate of the electron mediator – potassium hexacyanoferrate (III) – to the working electrode, resulting in a change in the current, which would indicate the presence of toxicants. The biosensor was used to test the toxicity of influents to WWTPs, and true-positive toxicity

signals were obtained from samples of influents to WWTPs receiving tannery industry wastewater mixed with domestic wastewater.

4.2.2. Microbial fuel cell (MFC) based biosensor

Bacterial oxidation of organic or inorganic compounds involves the transfer of electrons from the electron donors to the acceptors by a series of compounds called an electron transport chain. An MFC separates the electron acceptor from the biomass and shuttles the electrons through an electrical wire, therefore generating electricity while degrading the compounds. An MFC biosensor correlates the current or potential signal with substrate concentration. The MFC biosensor was first developed by Karube et al. [140] for the 5-day BOD measurement. Unlike the current setup, the bacterial cells in this prototype system were immobilized on a platinum anode. *Clostridium butyricum* cells were suspended in acrylamide monomer solutions and cast inside a nylon membrane which surrounded the anode. The biosensor needed a response time (time to gain a stable baseline between sample changes) of 20-30 minutes depending on the concentration of substrates. With real industrial wastewater, the current from the biosensor correlated linearly with BOD up to 300 mg/L; the measured BOD values were within the 10% error compared to standard readings. The current output was reproducible for 30 days and remained stable for 40 days. Since then a range of MFC biosensors have been developed, but the majority of them still target BOD measurement [132,141–143].

Recently, MFC biosensors for the measurement of VFAs and toxicity have shown potential to be used in the monitoring of biological WWTPs. Kaur et al. [144] investigated the use of MFC biosensors for specific VFA analysis; three H-type MFCs were inoculated with anaerobic digestion sludge, and fed with acetate, propionate or butyrate as the sole carbon source in a fed batch mode. The results showed that the peak currents in cyclic voltammetry scanning mode increased proportionally to the VFA concentrations up to 40

mg/L (10 mg/L for acetate) for the MFC fed with the same VFA. In another study, Liu et al. [145] developed a novel wall-jet MFC to monitor an upflow anaerobic fixed-bed (UAFB) reactor. The MFC sensor featured a sandwich layout of anode, proton exchange membrane, and cathode in a small chamber of 1.6 mL, and was inoculated with the biofilm of an old MFC that had been running for one year. Installed on the recirculation loop of the UAFB reactor and operated continuously, the output potential signals of the MFC biosensor corresponded well with the biogas flow rate at transient organic loads of synthetic wastewater. When real wastewater from a hydrolysis reactor was fed to the UAFB, the output potential also corresponded well with the biogas production rate, soluble and total CODs, total VFAs and acetic acid concentrations.

Dávila et al. [146] developed a microfabricated MFC biosensor of silicon wafers with arrays of channels of 40 µm x 40 µm x 300 µm in thecenter, which were deposited with Ti/Ni/Au sputtered tri-layers. The two electrodes were placed together with a proton exchange membrane sandwiched between them. The compact design of the MFC biosensor greatly reduced the distance between the electrodes, and distance for proton exchange, thus reducing the internal resistance of the cell to a minimum, and resulting in a maximum power density much higher than that achievable with a bigger setup. With constant current, the voltage output of the MFC-biosensor decreased immediately when formaldehyde was added to the anode compartment. Wang et al. [147] investigated the current changes of a single chamber bioelectrochemical cell with different concentrations of formaldehyde, and found a linear correlation up to 0.08% formaldehyde. In another set-up, Shen et al. [148] applied the MFC-biosensor for the toxicity measurement of Cu(II). In the continuous operation model, the MFC immediately detected toxicity when 5 or 7 ppm of Cu(II) was spiked into the feed of a real wastewater. They also investigated the effects of shear force on the anode-oxidizing biofilm and the sensitivity of the biosensor to toxicity, and found that a lower shear force

could increase the sensitivity of the biosensor. This finding matches with the phenomena observed by Patil et al. [149], as greater shear forces will result in the electroactive biofilm, which is more resistant to toxicants compared to planktonic organisms, as the dominant organisms in the anode chamber. Kim et al. [150] applied MFC-biosensors to monitoring toxicity in real wastewater, although during the one-year test there were no significantly toxic compounds present in the influent. However, when 0.01 mg/L Cd and 0.1 mg/L Pb were injected into the influent as artificial toxicants, the current dropped immediately. This suggested the suitability of employing an MFC-biosensor for toxicity monitoring; they also suggested diverting the toxic influent to a parallel MFC-biosensor in real situations to ensure that the biosensor could recover after exposure to the toxic compounds.

Both electroactive biofilms and planktonic mediator-assisted cells are widely used as components of MFC biosensors [141]. A recent study [149] investigating the sensitivity of anode microorganisms (both electroactive biofilms and planktonic cells) to toxins revealed some interesting phenomena. In the test, the electroactive biofilms were highly resistant to toxicants including sulfamethoxazole, sulfadiazine, chloramines B, Cu^{2+}, Ag^{+}, Pb^{2+} and Hg^{2+}, at concentrations similar to or higher than those in real wastewater, and hence no significant changes in currents were observed. Instead, the mediator-based planktonic MFC biosensor displayed reducing currents with the increase in toxicant concentration; these results suggested that for measuring toxicity, the anode microorganisms need to be carefully selected and validated. Sometimes, MFC biosensors inoculated with known mixed cultures were used [151] with the aim of reducing the uncertainty and enhancing long-term stability.

The most straightforward and widely-used way to quantify substrate concentrations from the signals of an MFC-biosensor is the linear relationship between stable currents and substrate concentrations [144,152,153]. To maintain a good baseline and prevent a false-positive measurement, the anodic potential of an MFC biosensor should be maintained within

a suitable range, and the pH has to be well controlled as well [154]. However, the fluctuations and interference caused by different substrates may make it very difficult when real wastewater samples are being analyzed. In order to overcome this limitation, a non-linear programming method integrated with an artificial neural network (ANN) was used to analyze the signals from MFC biosensors [155–157]. The ANN method utilized the information of the peak area, peak height, acceleration rate and subsidence rate of the current signals. After training, the MFC-biosensor could correctly identify four different substrates: acetate, butyrate, glucose and corn starch, when the four substrates were fed into the biosensor in a random sequence [156]. Furthermore, the COD measured with the MFC-biosensor correlated very well (R^2=0.99) with measurements based on the Standard Method [155].

4.2.3. Full performance reactor-based biosensors

Due to the complexity of sludge, a biosensor based on one single microbial strain, or a sub-group of sludge organisms, can easily trigger false-positive or false-negative signals. Thus, the best biosensors are those that make use of the whole sludge as the signal recognition element and subjected to a similar reactor setup. The problem, however, is how to achieve sufficiently prompt detection of toxicity in this complex biosensor which is mimicking the community in the sludge. Generally, two approaches have been used to solve this: measuring multiple parameters, e.g., DO and pH [158,159], and maintaining a higher metabolic activity in a full performance reactor-based biosensor [160–162]. These examples will be discussed in more detail here.

Kong and colleagues [163] described a RODTOX (Rapid Oxygen Demand and TOXicity tester) respirometric biosensor, which was an on-line monitoring set-up for BOD measurement in a small reactor (10 L). To make a measurement, the sensor was first calibrated with a pulse dose of substrate (150 mg/L COD of acetic acid), following which DO changes could be monitored. The DO depletion peak could be integrated online to provide

information about the peak height, peak area and peak slope. When the DO had returned to its normal value, the toxic compound was further added to the sensor in a pulse dose, and the DO depletion profile was retrieved again from the on-line DO data. The inhibition profile was obtained via comparison of the different DO depletion peaks. The biosensor can also be used to determine the inhibition to heterotrophic or autotrophic organisms [158].

Recently, a novel biofilm reactor (BFR)-based BOD biosensor was developed by Liu et al. [20]. The BFR was built with glass tubing (ø 0.2 mm × L 1.05 m), which was fed with air-saturated activated sludge to form the biofilm. Instead of using a conventional dissolved oxygen (DO) electrode to measure the BOD, the novel reactor was incorporated with a photoelectroncatalytic electrode, which could generate signal currents that correlated linearly with the COD levels. The COD changes after the samples were passed through the BFR were then converted to BOD values. The biosensor can analyze one sample in less than 15 minutes and give rise to a linear BOD range from 10 mg/L up to 800 mg/L. The biosensor remained quite stable for 30 days, suggesting that it may be possible to apply it to achieve toxicity monitoring of activated sludge.

Hong et al. [159] developed a DO- and pH-based biosensor for use in a nitrification inhibition assay. Two 3-L sequential batch reactors, equipped with a DO or pH probe, respectively, were used as the sensors with nitrifying activated sludge as the biological recognition element. A data processing algorithm was applied to log the change in pH and DO (i.e., dpH/dt and dDO/dt) every 1 min from the moving averages of readings of the two sensors. From the pH reading, the occurrence of an ‘ammonia valley (AV)’, which was the minimal pH attained due to the oxidization of ammonium, was determined. When the occurrence time of AV was 110% longer than the previous value, or when the OUR was 80% less than the previous value, a toxicant alarm in the samples will be triggered. The authors

successfully detected 2.5 mg/L Hg^{2+}, 0.5 mg/L allythiourea, and 0.25 mg/L chloroform through this system, and to finish one batch test it took about 60-70 minutes.

In another study, a 0.7-L (effective volume) anaerobic granular reactor based biosensor (AGB) was used as a biosensor for toxicity for both carbon- and nitrogen-oxidizing activated sludge in an aerobic reactor [164]. When phenol and Cu^{2+} were fed simultaneously to the aerobic reactor and the AGB, the oxidation-reduction potential (ORP) in the AGB corresponded with the variations of COD and NH_4^+ in the aerobic reactor. The response of the AGB manifested 6-20 h earlier than the aerobic reactor. A new on-line toxicity analyzer developed by LAR AG [165] utilized a nitrifying bacterial culture (*Nitrosomonas*) to detect toxicity. The respiration inhibition of the self-regenerating culture was monitored and provided toxicity measurement with 5-10 minutes.

A novel nitrous oxide accumulation based biosensor was proposed by Black et al. [166]. Instead of monitoring nitrification in WWTPs, the proposed biosensor measured nitrous oxide emissions in a sewer pipe upstream of WWTPs. After a shock load of the toxicant of allylthiourea to the real wastewater for 90 minutes, the nitrous oxide level in the pipes tested were higher than the control for the following 12 hours. However, when a shock load of potassium dichromate, copper sulphate, and nickel sulphate were applied to the system, the increase in nitrous oxide in the test samples was not obvious. Sometimes the control even had a higher gas level, and in addition, the control baseline fluctuated a lot with real wastewater, which was very challenging for data processing.

Rozzi and his colleagues [160–162] did some pioneering work on toxicity measurement in anaerobic digestion, and developed a Rantox (Rapid ANaerobic load and TOXicity test) biosensor. The Rantox was a small anaerobic digester which was installed online before the wastewater was directed into the big anaerobic digester. The Rantox sensor was inoculated with the same sludge as that of the big reactor, and was also fed at the same

organic loading rate. The only difference was that acetic acid was fed into the Rantox at an auto-adjustable frequency to keep the specific biogas production rate at the maximum level. The concept is that inhibition of methanogens caused a decrease in the specific substrate utilization rate, which in turn will result in the accumulation of substrate. Based on the Monod equation, these two factors present opposing effects on the specific substrate utilization rate, and hence the inhibitory effects were hard to detect in the beginning. However, in the Rantox the substrate concentrations were kept at a greater-than- saturation level, and thus the inhibitory effects could easily be detected from a decrease in the specific substrate utilization rate (or specific biogas production rate). In Rantox, the biogas flow rate and pH were continuously measured and the maximum biogas production rate was continuously calculated using software and this was plotted. In a test with yeast-factory wastewater, the Rantox signals were shown to have very good reproducibility [161]. For three different sludge sources, when the Rantox was tested with chloroform it detected the toxicity (5-10% decrease of the biogas production rate) within three cycles of acetate spike (3 hours per cycle) at an HRT of 10 hours. At the same time, the inhibitory effects to sludge in big digesters was not observed when using the Rantox sensor [162].

However, the Rantox did not differentiate between total biogas and methane, which could cause some uncertainties in measurement. Hence this same group developed a titration bioassay to determine methanogenic activity [162]; acetate was used in a batch bioassay reactor, and the pH was monitored. When acetate was consumed, concentrated acetate was titrated into the reactor to keep the pH constant, and the acetate added equaled the acetate consumed by methanogens; thus the titrant volume can be used to calculate methanogenic activity. A test with chloroform displayed very quick measurement (40 minutes) of inhibition in terms of specific methanogenic activity: although the method is promising, more work

needs to be done to decrease the uncertainties in the interpretation of diagrams and to provide quantitative criteria for inhibition.

Oh et al. [167] developed a new biosensor for monitoring the toxicity of Cr (VI) in water by using changes in the electrical conductivity (EC) and pH caused by the inhibition of sulfur-oxidizing bacteria (SOB). The SOB were grown in a 100 mL continuous reactor packed with sulfur particles, which were converted to sulfate and protons through the biological activity of SOB. With an HRT of 30 minutes, the differences in EC and pH values between the influent and effluent could be determined. When the toxicant - Cr (VI) at a concentration as low as 5 ppb was fed in the influent, the change of EC and pH values in the effluent could be determined after 1 hour. The detection limit of this method was much lower than the common bioluminescent assay, and this technique was used successfully in the toxicity assessment of industrial effluents containing 1,4-dioxane [168].

5. Comparison of different assays and future research needs

As discussed in an earlier section, there are two types of toxicity assays: the batch-based direct measurement of toxic effects on the native biological community; and, the indirect measurement of toxic effects on bioluminescent indicator organisms or biosensors. The two types of toxicity assays have their own application niches: the batch-based direct measurement is more applicable when a new chemical or a new wastewater has to be evaluated for basic toxicity. The indirect measurement is more suitable as a front-line screening device to protect an operating WWTP. A comparison of the above-mentioned toxicity assays is listed in Table 2.

Recently, both the direct and indirect toxicity assays have been used in full-scale biological WWTPs. A wastewater treatment facility receiving more than 85% of wastewater from industrial sources in Virginia, USA, had experienced a number of random toxic upsets [169]. Thus, an early warning system (EWS) based on ATP luminescence assay developed by

LuminUltra (Fredericton, New Brunswick, Canada) was implemented, which identified a significant toxic upset and facilitated troubleshooting after the upset. In another case, an EWS based on a full performance reactor-based biosensor - BioCoil^{TM}, which consisted of a small aeration tank and an unaerated plug-flow reactor, was successfully used over the past six years [170]. The pH, temperature, suspended solids, and dissolved oxygen were sensed in the EWS, and the OUR was determined continuously. The EWS successfully warned of a chemical spill incident, and helped to identify the source of the incident. These examples clearly demonstrate that an EWS based on an effective toxicity assay can protect WWTPs from toxic shocks. However, research is still needed in the following areas:

1) Dehydrogenase activity assay

OUR- or DO-based respiration inhibition assays and the ammonium-based nitrification inhibition assay have been incorporated into whole-cell biosensors. Amongst the rest of the batch-based assays reviewed in this paper, the dehydrogenase activity assay seems to be very promising for adoption as an on-line toxicity sensor due to its high correlation with the OUR assay, and its easy data acquisition via a fluorescence detector. However, the flocs and particles in sludge may interfere in the detection of the fluorescence; thus future studies should focus on how to eliminate this interference.

2) Bioluminescent assays and whole-cell biosensors

The bioluminescent assays, or whole-cell biosensors based on genetically modified strains of bacteria that originate from activated sludge, such as *Nitrosomonas* sp. and *Nitrobacter* sp. have the potential to be adopted in WWTPs. However, a single toxicant can cause different effects on different organisms; thus assays or sensors based on a specific organism may fail to trigger a toxic alarm. Before a bioluminescence assay or biosensor can be used in WWTPs, its sensitivity to a matrix of various toxicants has to be examined. If needed, a combination of different organisms in one sensor, or incorporation of multiple

sensors is a possible choice to provide full protection to WWTPs. The short life-time of a bioluminescent assay or whole-cell biosensor poses another challenge to its application as an on-line toxicity tool. The method for *in-situ* cultivation of the reporter organisms has to be improved before these sensors can be used as on-line tools. Despite the above challenges, the bioluminescent assay or biosensors with the species- or effect-specific reporter genes fused are the best fit for an emergent toxicity analysis to identify a potential toxicant, or the specific effect that a toxicant causes.

3) MFC- and full performance reactor-based biosensors

The MFC- and full performance reactor-based biosensors have the advantages of medium sensitivity and a quick response that is likely to be sufficient to protect the integrity of most WWTPs. Both of them are almost free from the need to constantly maintain the indicator bacteria. The MFC biosensor has a quicker response than the full performance reactor biosensor, although the latter has the advantage that it can provide detailed information about which specific process is inhibited.

However, the correlation of MFC-based biosensors with the traditional OUR-based assays in response to real wastewaters has to be investigated carefully before they can be used in biological WWTPs due to the difference between the anode microorganisms and sludge in WWTPs. However, a full performance reactor-based biosensor has been used successfully in a full-scale WWTP. The bigger working volume of such a biosensor better mimics a full-scale WWTP, thus resulting in more reliable results, although the increased maintenance work induced by their bigger volume prohibits their wide application. Thus, research on reducing the effective volume of a full performance reactor-based biosensor while keeping its sensitivity is needed.

4) Bioassay/biosensor specific for anaerobic processes

Currently the CH_4 production-based ATA is widely used. However, unlike the OUR-based respiration inhibition test, which has been rigorously standardized for aerobic processes over the past decades, there has been to date, no established standard method for anaerobic CH_4-based ATAs, which makes it very difficult to compare results across different labs. Thus, effort needs to be made by the scientific community to standardize the ATA.

With the advancement of membrane technology, the application of anaerobic processes as a stand-alone technology for full-scale WWTPs is very attractive. However, biosensors specific for anaerobic processes are not available yet. One major difficulty of adopting the CH_4 production-based ATA as an online tool is that the change of methane content in biogas is not significant and hard to detect right after the toxicants are introduced to the influent. In contrast, MFC-based biosensors are very promising for anaerobic processes due to their quick response, long-term stability and robustness. However, the difference between the anode microorganisms and anaerobic digestion sludge will inevitably result in different toxic effects, which have to be examined carefully in labs and in pilot-scale WWTPs.

5) On-line data processing method

A lot of effort has been put in to develop novel biosensors. However, how will these biosensors perform in full-scale biological WWTPs? The fluctuations in influent quality and quantity cause large uncertainties in the data obtained from the biosensors. This was highlighted in a recent report by WERF [171] documenting a case study of an EWS in a WWTP to prevent upsets caused by chemical/biological/radioactive contaminants: the key issues for a robust EWS identified by this project were the data quality and real-time data analysis. Therefore, advanced data processing methods, which can differentiate between true- and false-positive signals, are needed for an efficient EWS in real WWTPs. The ANN

method used in MFC biosensors [155–157] is very promising, although future studies with raw wastewaters in laboratory- or pilot-scale experiments are needed.

6. Conclusions

Different toxicity assays are available for biological WWTPs; amongst them, the OUR-based respiration inhibition test, and the CH_4-based ATA assay are the most widely used in aerobic and anaerobic processes, respectively. Standardization of the CH_4-based ATA assay is urgently needed in order to compare results across different labs. Recently, EWs based on reliable on-line toxicity biosensors have provided protection from toxic shocks to WWTPs. However, more research is needed to: test the response of bioluminescence assays and whole-cell sensors based on specific organisms to various toxicants, and build a matrix of biosensors or a biosensor of multiple reporter organisms that can protect the integrity of WWTPs; correlate the MFC-based biosensors with the traditional OUR-based or CH_4-based assay in response to real wastewaters; reduce the size of full-performance reactor-based sensors; and, develop more advanced on-line data processing methods to correctly interpret the results obtained from toxicity sensors in real WWTPs.

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Table 1 Approaches to measuring toxicity in biological wastewater treatment processes

	Reactant consumption	Indicator of organism properties	Product generation
Oxygen uptake rate (OUR)	✔		
CO_2 production			✔
NH_4^+ consumption	✔		
NO_2^- production/ consumption	✔		✔
NO_3^- production			✔
N_2O production			✔
CH_4 production			✔
Volatile fatty acid (VFA) accumulation			✔
Cell density		✔	
Cell viability		✔	
Floc morphology		✔	
Enzymatic activity		✔	

Table 2 Comparison of different toxicity assays

Measurement method	Exposure time	Target organisms	Sensitivity	Key features
		Direct measurement		
OUR based respiration inhibition	30 min – 3 h	Aerobic heterotrophs and chemoautotrophs	Middle	1. Standard method, easy to compare results between different labs. 2. Target whole activated sludge, the results can be applied directly to real process design. 3. Relatively short incubation time.
CO_2 evolution test	7 d – 28 d	Aerobic heterotrophs and chemoautotrophs	Middle	1. Results indicative of the biodegradability of target chemicals. 2. Long incubation time.
Nitrification inhibition	4 h – 3 d	Aerobic chemoautotrophs	High	1. Sensitive, criteria based on this result can protect the whole activated sludge. 2. Medium incubation time.
CH_4 production ATA assay	5 d – months	Methanogens	High	1. Very sensitive, criteria based on this result can protect both the activated sludge and anaerobic digestion sludge. 2. Target on anaerobic sludge, the results can be applied directly to real process design. 3. Very long incubation time. 4. Not standardized, difficult to compare results across labs.
Enzymatic activity	30 min – 2 h	Any microorganisms	Middle	1. Generic to both anaerobic and aerobic sludge. 2. Short incubation time. 3. No indication of which process is inhibited.
Cell growth	1d – 5 d	All, but only for pure cultures in practice	Middle	1. Growth rate can be determined at the same time. 2. Not suitable for mixed cultures or sludge.
Mortality – CFU based	1 d – 3 d	All, but only for pure cultures in practice	Middle	1. Widely used for pure cultures 2. Not suitable for mixed cultures or sludge
Cell membrane integrity assay	15 min – 7 h	Any microorganisms	Middle	1. Easy to employ (for staining kit) 2. More specific for cytotoxicity assay 3. No indication of toxicity to the process.
Floc morphology	1 h –	Activated sludge	Low	1. Easy to employ.

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	7 h			2. Needs experienced technologist to interpret the results. 3. Not widely used for anaerobic processes, especially difficult for granules.
			Indirect measurement	
Bioluminescence assay	5 min – 30 min	Indicator organism	Very high	1. Very sensitive, but maybe too sensitive for sludge so provides false-positive results. 2. Can be used either in batch or on-line continuously. 3. Very quick response time 4. Standard methods, easy to compare results across different labs. 5. No indication of toxicity to processes. 6. Colors and particles in the influent interfere with the assay. 7. Difficult to maintain the reporter bacteria.
Whole-cell biosensor	~ 30 min	Indicator organism	High	1. Sensitive. 2. Can be used either in batch or on-line continuously. 3. Quick response time 4. No indication of toxicity to processes. 5. Short lifetime.
MFC based biosensor	30 min - 1 h	Indicator organism	Middle-High	1. Sensitive. 2. Can be used either in batch or on-line continuously. 3. Quick response time 4. Almost free of maintaining the reporter bacteria.
Full performance reactor based biosensors	1 h - 9 h	All	Middle-High	1. Results highly relative to either anaerobic or aerobic processes depending on the sensor used 2. Can be used either in batch or on-line continuously. 3. Quick response time. 4. Almost free of maintaining the sensing bacteria. 5. Needs to be validated for each process and different sludge sources.