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Rapid BOD assessment with a microbial array coupled to a neural machine learning system

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ABSTRACT

The domestic usage of water generates approximately 310 km^3 of wastewater worldwide (2015, AQUASTAT, Food and Agriculture Organization of United Nations). This sewage contains an important organic load due to the use of this water; this organic load is characterized using a standard method, namely, the biological oxygen demand measurement (BOD_5). The BOD_5 provides information about the biodegradable organic load (standard ISO 5815). However, this measurement protocol is very time-consuming (5 days) and may produce variability in approximately 20% of results mainly due to variation in the environmental inocula.

To remedy these limitations, this work proposes an innovative concept relying on the implementation of a set of rigorously selected bacterial strains. This publication depicts the different steps used in this study, from bio-indicator selection to validation with real wastewater samples. The results obtained in the final step show a strong correlation between the developed approach and the reference method (ISO 5815) with a correlation rate of approximately 0.9. In addition, the optimization of the experimental conditions and the use of controlled strains (8 selected strains) allow significant reduction in the duration of the BOD_5 analysis, with only 3 h required for the proposed method versus 5 days for the reference method. This technological breakthrough should simplify the monitoring of wastewater treatment plants and provide quicker, easier and more coherent control in terms of the treatment time.

1. Introduction

The global production of domestic wastewater was assessed to be 315 km^3 in 2010 and will tend to increase in the future concomitant with population expansion (Flörke et al., 2013; Mateo-Sagasta et al., 2015). These wastewaters, which are mainly contaminated by organic materials (i.e., carbohydrates, fats, proteins, etc.) resulting from human body wastes (20% - faeces and urine) and domestic uses (80% - personal washing, laundry, food preparation, etc.) (Duncan, 2004; Garcia et al., 2006) present a real risk for the receiving environment and the exposed population. To limit the consequences of release of these wastewaters into the environment, they are treated to remove the organic load through bioprocesses, such as wastewater treatment plants (WWTPs). To measure the conformity of the released waters, the organic load in the treated water is tested weekly in average samples according to the reference method (ISO 5815–1:2003, 2003; ISO 5815–2:2003, 2003) based on BOD (biological oxygen demand).

This method dates from the beginning of the twentieth century (Great Britain. Royal commission on sewage disposal, 1908; Jouanneau et al., 2014), and its implementation is widespread worldwide. This method is based on assessment of the biochemical oxygen demand [i.e., the amount of oxygen consumed over 5 days by environmental microorganisms to biodegrade the organic matter under aerobic conditions (BOD_5 in $mg.L^{-1}$ of consumed O_2)]. However, this method has several drawbacks:

- The method exhibits important intrinsic variability (higher than 20%) that is mainly due to the broad diversity of inocula used to carry out the measurements (Guyard, 2010).
- The 5-day period required for a measurement is longer than the retention time of water in WWTPs (close to 3 days).
- The measurement carries a significant risk of measurement error due to the available amount of oxygen in water. Indeed, the maximal load measured with this method cannot exceeded

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6 mg/L BOD (ISO 5815−1:2003, 2003; ISO 5815−2:2003, 2003), whereas the BOD_5 value of wastewater is close to 300 mg/L and thus requires pre-dilution.

Over the last several decades, many research teams have focused on these issues to propose new measurement strategies with an aim of removing these drawbacks (Jouanneau et al., 2014; Sun et al., 2015; Yang et al., 2015). A specific focus on the biological aspect of the measurement highlights two strategies based on (i) the use of environmental inocula and (ii) the implementation of only one microbial strain. The first strategy has the advantage of ensuring great representativeness of the biological answers due to broad microbial diversity but induces significant variability (i.e., origin, density, and diversity) (Liu et al., 2012; Rastogi et al., 2003; Rocher et al., 2011) due to the intrinsic characteristics of the inocula. Conversely, the second strategy, based on the use of one known strain, is very reproducible but is limited by the range of metabolic abilities of the selected strain (Arlyapov et al., 2012; Raud et al., 2012; Yoshida et al., 2001), which consequently provides a truncated view of the biodegradable organic diversity.

In light of these issues, some researchers have proposed an alternative strategy based on the use of several known microbial strains. Two strategies were proposed based on either (i) an artificial mixture of 2−4 strains or (ii) a set of several strains used individually. The aims of these strategies were the same, namely, to enlarge the biodegradation spectra while ensuring a great level of reproducibility (Catterall et al., 2003; Jia et al., 2003; Jiang et al., 2006; Lin et al., 2006; Xin et al., 2007). In the case of multi-species mixtures (i), the stability of the initial properties of the consortia (i.e., cell density, diversity, ratio between strains, biodegradation abilities, etc.) was difficult to ensure long-term mostly due to competition for the carbon substrate. The second strategy (ii) (Pitman et al., 2015; Raud and Kikas, 2013) also relies on several strains but is based on the use of several selected strains (not structured in the consortium). This strategy ensures good reproducibility while freeing the results from potential inocula drifts (this strategy was applied in this work). Nevertheless, this multivariate approach (based on several biological descriptors) increases the complexity of the data analysis (one value per strains). To overcome this limitation, Raud and Kikas (2013) analysed the data with a PLS (partial least squares) regression method to simultaneously take into consideration all the information provided by the different strains. The results were very encouraging, but the validation was performed only on synthetic wastewaters and not on real samples.

Moreover, bio-element selection is a crucial parameter in these metrological approaches based on controlled strains and directly conditions the potential performances of the future analytical method (i.e., biodegradation spectra and response kinetics) (Jouanneau et al., 2014). The selection step, when included, generally is based on either the intrinsic properties of the strains (i.e., biodegradation range and growth conditions) (Kwok et al., 2005; Oota et al., 2010; Raud et al., 2012; Raud and Kikas, 2013) or isolation of strains from a specific environment (Chan et al., 2000; Kwok et al., 2005; Yoshida et al., 2000). Nevertheless, no characterization or comparison has been carried out to determine the relevance of this choice. Moreover, questions remain concerning how to ensure the representativity of the proposed analytical approach under these conditions.

The aim of the proposed study was to design a relevant analytical method for the assessment of the BOD_5 to address these limitations. This strategy is based on the separate use (not structured in the consortium) of a set of 8 bacterial strains that have been rigorously selected using defined characteristics, such as the biodegradation potential, metabolic rate and toxicological robustness (Fig. 1). Coupled with an algorithm of data processing allowing to analyses the multivariate information provided by the bacterial set, the proposed method is able to assess BOD_5 in only 3 h with a high reliability ($r^2 = 0.85$).

2. Experimental section

2.1. Bacterial strains

A total of 28 strains were preselected from the literature according to the following characteristics: (i) their representativity with regard to the microbial population of an activated sludge from a WWTP (Kong et al., 2007; Snaidr et al., 1997; Xia et al., 2010), (ii) their weak pathogenicity (mainly group 1 risk according to the 2000/54/EC classification proposed by the European Directive), (iii) their broad biodegradation abilities (Gao et al., 2010) and (iv) their resistance to freeze-drying conditions (information collected from microbial collections, such as ATCC, DSMZ or CIP) (specification relative to an eventual industrial transfer). The full list of pre-selected strains at this step is described in the supplementary data (Tables SD−1).

2.2. Growth medium of the strains and other solutions

The Luria Bertani medium (LB medium) was prepared in distilled water and supplemented with 5 $g.L^{-1}$ NaCl (Merck, Germany), 5 $g.L^{-1}$ tryptone (Biokar Diagnostics, France) and 1 $g.L^{-1}$ yeast extract (Biokar Diagnostics, France). 15 $g.L^{-1}$ of agar type E (Biokar Diagnostics, France) was added to obtain the solid medium. The pH was adjusted to 7 using a solution of HCl (Sigma Aldrich, USA) or NaOH (Merck, Germany) before autoclaving at 120 °C for 20 min.

The synthetic wastewater (SWW) solution was prepared in accordance with the OECD 209 standard[28] as follows: 1 L of distilled water was supplemented with 16 g of tryptone (Biokar Diagnostics, France), 11 g of meat extract (Biokar Diagnostics, France), 3 g of urea (Sigma-Aldrich, USA), 0.7 g of NaCl (Merck, Germany), 0.4 g of $CaCl_2 \bullet 2H_2O$ (Merck, Germany), 0.2 g of $MgCl_2 \bullet 7H_2O$ (Sigma-Aldrich, USA), and 2.8 g of K_2HPO_4 (Merck, Germany).

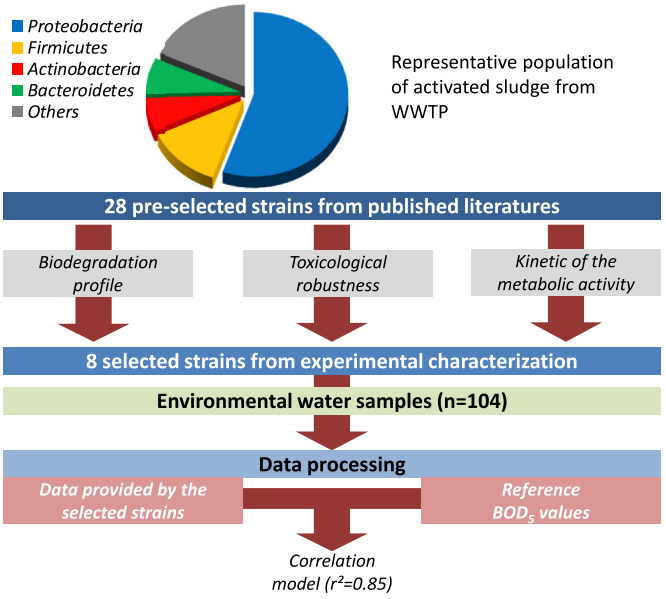

Fig. 1. Strategy applied to the development of the BOD_5 assessment method (Isazadeh et al., 2016; Xia et al., 2010; Zhang et al., 2018).

The mineral medium [MM, derived from M9 medium (Atlas, 1997)] without a carbon source used in this study was prepared in two steps. The first step consisted of preparation of several concentrated solutions (S1, S2, S3, St and Sb) in distilled water. S1 consists of 60 g.L^{-1} Na_2HPO_4 (Fluka, Germany), 30 g.L^{-1} KH_2PO_4 (Merck, Germany), 10 g.L^{-1} NH_4Cl (Merck, Germany) and 5 g.L^{-1} NaCl (Merck, Germany). S2, S3, Sb and St are composed of 264.5 g.L^{-1} $MgSO_4 \bullet 7H_2O$ (Sigma-Aldrich, USA), 14.7 g.L^{-1} $CaCl_2 \bullet 2H_2O$ (Merck, Germany), 0.5 g.L^{-1} biotin (Sigma-Aldrich, USA) and 0.15 g.L^{-1} thiamine • HCl (Sigma-Aldrich, USA), respectively. S1, S2 and S3 were sterilized by autoclaving, and St and Sb were filtered through 0.2 μm cellulose acetate filters (146560, Dutscher, France) and stored at +4 °C. The second step consisted of mixing these solutions according to the following ratio (for 1 L of MM): 100 mL of S1, 1 mL of S2/S3/St/Sb and if required 10 mL of a carbon source at the desired concentration. The mixture was adjusted to 1 L with sterile distilled water.

Glucose solutions (GSs) were prepared at several carbon concentrations in the MM medium and sterilized by filtration (cellulose acetate filters, 146560, Dutscher, France).

Glycerol solution (GlyS) at 50% (v/v) was prepared in distilled water and sterilized by autoclaving.

2.3. Growth and preparation of bacterial cells

All strains were stored and cultivated under the same conditions. The cells were stored at −80 °C in a mixture (v/v) of LB medium (50%) and GlyS (50%). The cells were isolated on solid LB medium at +30 °C during 12−24 h and the obtained colonies were used to inoculated the first cultivations.

For the first cultures, the cells were grown overnight (14 h) in LB medium at 30 °C under shaking at 250 rpm. Then, the cells were diluted to an optical density of $OD_{620nm} = 0.2$ in fresh LB medium and cultivated at 30 °C under shaking at 250 rpm until an $OD_{620nm} = 0.6$ (exponential growth stage) was reached. This OD_{620nm} value (0.6) corresponds to a cell density varying between 10^8 and 10^9 CFU ml^{-1} for the tested strains (results not shown). For the sake of simplicity, we considered that these cell densities were not significantly different from strain to strain.

The cultivated cells ($OD_{620nm} = 0.6$) were refreshed in an ethanol bath at −20 °C for 1 min. The aim of this step is not to freeze the cells but to decrease quickly the temperature in order to avoid any change of their physiological state. Then, the cells were centrifuged (6400×*g*, 5 min, +4 °C) to remove the growth medium and resuspended in a cold washing solution of $MgSO_4 \bullet 7H_2O$ (10^{-2} M). After three washes, the cells were resuspended in MM without a carbon source and adjusted to obtain the required cellular density ($OD_{620nm} = 0.43/0.86/1/1.72/2.57/3.43/4.29$).

2.4. Optimization of the experimental conditions

We were interested in improving some of the experimental conditions through two strategies: the replacement of oxygen (used as an activity marker of biodegradation in the reference method) with a fluorescent dye (resazurin) that was *a priori* better suited for this metrological context and the optimization of the cell density in the biodegradation assays. The aim of this second strategy was to define the condition that ensured both a reduced analysis duration and a measurement range that was as large as possible. The resazurin solution used in this work was a commercial solution (PrestoBlue®, Invitrogen™, USA). No information was available relative its concentration.

These steps were performed with the model strain *E. coli* K12 MG1655 in duplicate.

2.4.1. Comparison of two activity markers: oxygen versus resazurin (fluorescent dye)

2.4.1.1. Monitoring the biodegradation activity via the fluorescent marker. The bacterial suspension was prepared as described above (see 2.3) at an $OD_{620nm} = 0.43$. The assay solution was prepared by mixing the washed bacterial suspension, MM supplemented with glucose and the commercial resazurin solution (PrestoBlue®, Invitrogen™, USA).

Several glucose concentrations (final concentration) ranging from 0.1 mg.L^{-1} to 500 mg.L^{-1} were tested to define the limits of the measurement range. The mixtures were prepared directly in the microplate wells (Nunc™ F96 MicroWell™, 236108) in following the ratio (final volume = 150 μL/well): 35 μL of bacterial suspension (final $OD_{620nm} = 0.1$), 100 μL of MM with glucose and 15 μL of dye (PrestoBlue®, Invitrogen™, USA, in accordance with the provider protocol: 10% v/v).

Monitoring of the biological activity via the fluorescent marker (Cregut et al., 2013; Dudal et al., 2006; Tizzard et al., 2006) was performed with the TriStar LB 941 Multimode Microplate Reader (Berthold Technologies GmbH, Germany) every 10 min [$\lambda_{excitation} = 540$ nm (bandwidth = 10 nm), $\lambda_{emission} = 610$ nm (bandwidth = 10 nm), counting time = 0.1 s and incubation temperature = 30 °C].

2.4.1.2. Monitoring of the biodegradation activity via oxygen consumption. For the assay based on the oxygen marker, the resazurin solution was replaced by distilled water in the assay solution. In this case, the biological activity was monitored in an Oxodish® OD24 microplate reader (24-well microplate with an integrated oxygen sensor, PreSens Precision Sensing, GmbH, Germany). Each well was filled with 3 mL of assay solution (same ratio as in 2.4.1.(a) − 700 μL of bacterial suspension, 2 mL of MM with glucose but 300 μL of distilled water) and sealed using an AB-0558 adhesive PCR film (Thermo, ABgene, France) to prevent atmospheric oxygen from entering the system (Cregut et al., 2013) during the test. Oxygen was monitored at 30 °C with the SensorDish Reader (PreSens Precision Sensing).

2.4.2. Optimization of the cellular density

The impact of the cell density was evaluated as described in the previous paragraph (2.4.1.). The same protocol based on resazurin as the biodegradation marker was used except for the final bacterial concentration, which ranged from $OD_{620nm} = 0.1$ to $OD_{620nm} = 1$.

2.5. Characterization of the strains

The characterization steps described below were performed for the 28 selected strains in parallel with the optimization phases. The cellular density after the washing step (see 2.3) was adjusted to $OD_{620nm} = 1$ for all strains.

2.5.1. Metabolic profile and stress robustness

The objective was to characterize the selected strains according to two criteria: their biodegradation capabilities and their intrinsic robustness towards several environmental stresses (i.e., pH, salinity, antibiotics, reference toxic compounds, etc.). These two parameters were assessed simultaneously using GEN III microplates™ (Biolog Inc., USA − see Fig. SD-1) with 71 different carbon sources and 23 stress conditions (test based on the growth inhibition induced by specific conditions such as osmotic and pH stresses or several reference toxics) supplemented with tetrazolium redox dyes (as a colorimetric respiratory marker) following the protocol provided by the manufacturer.

Briefly, after the washing step (see 2.3), the bacterial suspension was diluted tenfold in an IF-A solution (Biolog Inc., No. 72401, IF-A,

USA) to obtain a cellular density between 10^7 and 10^8 cell.mL^{-1} ($OD_{620nm} = 0.1$). Then, 100 μL of this diluted suspension was added to each microplate well, and the plate was incubated for 40 h at 30 °C. The reading was performed at a wavelength of 590 nm (Preston-Mafham et al., 2002) with the TriStar LB 941 multimode microplate reader (Berthold Technologies, Germany). Each strain was tested in duplicate.

The results obtained from the 71 carbon sources were used to establish biodegradation profiles specific to each strain and then compared and clustered using statistical approaches (agglomerative hierarchical clustering, XLStat, Addinsoft).

For each strain, an index of robustness was calculated from the number of stress conditions, conditions inducing a growth inhibition (none observed cellular development), divided by the overall number of tested conditions (n = 23). This index aims to compare the sensitivity of the strains to potential environmental stresses.

2.5.2. Assessment of the biodegradation kinetics

The biodegradation kinetics of the 28 strains were compared in the SWW medium to evaluate their respective response times. First, 2.7 mL of SWW and 300 μL of the washed suspension ($OD_{620nm} = 1$) were added to the wells of an Oxodish® OD24 microplate (PreSens, Germany). An AB-0558 adhesive PCR film (Thermo, ABgene, France) was applied on the microplates to limit atmospheric oxygen diffusion in the reaction medium. Measurements were performed with the SensorDish Reader (PreSens Precision Sensing) each minute at 30 °C for 10 h in triplicate. The kinetics values were calculated from the maximal slope of the measured oxygen consumption.

2.6. Environmental samples

The environmental samples (n = 104 - Tables SD−2) had several origins, including raw or treated waters from several WWTPs in the Nantes countryside (France, n = 72), stormwaters from the Bordeaux Region (France, n = 8) and treated waters collected from non-collective treatment units (autonomous sanitation system dedicated to the isolated private dwellings) installed in the Scientific and Technical Centre for Building of Nantes (CSTB, France, n = 24). The sampling procedure used for this study follows the conditions described by the ISO 5667-2 and ISO 5667-3 standards. The samples were collected in 250 mL disposable plastic bottles (PE, 030424, Dustcher, France) that were previously washed with distilled water. The samples were aliquoted into 2 mL tubes (PP, BiopurTM Safe-LockTM tubes, Eppendorf®, Germany) and stored at −20 °C prior to use.

Several parameters were measured to characterize the samples, including the BOD_5 (see 2.7), chemical oxygen demand (NF T90-101/NF EN 872), and toxicity (ISO 11348, ISO 6341 and ISO, 20666) (data shown in Tables SD−2).

2.7. BOD_5 measurement

2.7.1. Reference method (ISO 5815)

The reference analyses for BOD_5 were performed according to the standard method (ISO 5815) by the INOVALYS NANTES Lab (Nantes, France), which is certified by the French Accreditation Committee (COFRAC).

2.7.2. Method developed in this work

The measurement protocol for the proposed BOD_5 test was carried out in 96-well microplates (Nunc™ F96 MicroWell™, 236108) according to the following ratio: 35 μL of bacterial suspension at an $OD_{620nm} = 2.57$ (final OD_{620nm} in well = 0.6), 100 μL of sample and 15 μL of resazurin (PrestoBlue®, Invitrogen™, USA). The microplate was incubated for 3 h at 30 °C. These test conditions (incubation period, cell density, oxygen marker, and used strains) were determined during the optimization step of the experimental conditions (see results in 3.1 and 3.2). Subsequently, the fluorescence signal relative to the biological activity of the strains was individually read with the TriStar LB941 Multimode Microplate Reader as described above (see 2.4.1).

All the samples (see 2.6) were tested in duplicate, and the collected values were exported to an overall database. This data set was used to establish the correlation algorithm between the biological values provided by the bacterial set and the BOD_5 reference method (ISO 5815).

2.8. Data processing: design of correlation models by a statistical approach

The aim of the data processing was to design algorithms to correlate the biological data (fluorescence values) obtained from the selected strains with the BOD_5 reference values; the final goal was to propose a mathematical model to predict the BOD_5 value from only the data provided by these strains. All models were designed via a dedicated software (Neuro One 6.13.0.5, Netral, France).

2.8.1. Architecture of the model (number of biological descriptors and nature of the model)

First, the data were analysed using a mono-parametric approach. The information (fluorescence values) from each strain was individually correlated with the reference values by linear regression. Because the results were insufficient (low correlation rates), the data processing focused on a multi-component strategy (based on several strains), which was also based on a linear regression algorithm. This step allowed the determination of the number of biological descriptors (selected strain) required to assess the BOD_5. The predictive abilities of these models are limited.

To improve the performance of the models for the assessment of the BOD_5, non-linear algorithms by neural networks were deployed. These models are defined according to architectural characteristics, such as the number of hidden neurons, the activation modalities of the neurons, and the data standardization conditions. Several architectures were compared for the overall database to identify *a priori* the most relevant algorithms.

2.8.2. Model validation

The last data processing step consisted of comparing the preselected architectures based on their predictive abilities with new samples. For this purpose, the overall database was randomly separated into two groups: a learning set (n = 148 assays) and a validation set (n = 60 assays). The learning set was used to design new algorithms based on the preselected architectures (number of hidden neurons, activation mode, and data standardization). To assess the robustness of these new models and choose the most relevant model, the estimated BOD_5 values obtained from the validation set were compared to the reference values. This step was also performed with Neuro One 6.13.0.5 (Netral, France).

3. Results and discussion

3.1. Improvement of biological activity monitoring

The reference method used to assess BOD_5 is based on oxygen consumption as a marker of the biodegradation activity. This approach induces some technical constraints, such as a measurement range that is limited by the saturation rate of oxygen in water (Cregut et al., 2013) or the tightness of the system to air

(contaminant oxygen). In this context, resazurin appears to be a relevant marker for monitoring biological activity (Cregut et al., 2013). This non-toxic non-fluorescent dye ($\lambda_{absorbance\ max} = 601$ nm) is reduced in the cell cytoplasm to a fluorescent compound, resorufin ($\lambda_{absorbance\ max} = 571$ nm, $\lambda_{excitation} = 535-560$ nm, $\lambda_{emission} = 590-615$ nm – see Fig. SD-2), by the enzymatic chain involved in cellular respiration (Cregut et al., 2013; Fai and Grant, 2009; O'Brien et al., 2000).

These two cell activity markers were compared during glucose biodegradation monitoring (MM supplemented with glucose as the sole carbon source) in *E. coli*. Several glucose concentrations (range from 0.1 to 500 mg.L^{-1}) were tested to determine the highest measurable concentration according to the marker used (Fig. 2A). Comparative analysis of these results showed that use of resazurin as a marker could assess the glucose concentration up to 495 mg.L^{-1} versus only 125 mg.L^{-1} with oxygen. In light of these data, the fluorescent dye was selected for continuation of the study.

Subsequently, we were interested in the effect of the cell density of *E. coli* on the measurement duration with resazurin as cell activity marker. Fig. 2B shows that an increase in the cell density substantially reduced the analysis duration; a cell density of 0.1 (OD_{620nm}) resulted in a measurement duration of almost 12 h versus 2 h for an $OD_{620nm} = 1$. The results also showed a reduction in the measurement range that was linked to the increasing cell density. This trend can be explained by an increase in the signal-to-noise ratio (see Fig. SD-3). An important noise (fluorescence baseline) measured with the high cell densities probably is induced by endogenous cellular respiration. At the same time, the saturation limit of the approach is reached at approximately 800,000 RFU. Consequently, the maximal measurable concentrations should not exceed 500 mg.L^{-1} for a cell density of 0.1 (OD_{620nm}) versus 170 mg.L^{-1} for an $OD_{620nm} = 1$.

To comply with the approach expectations (analysis limited in time but ensuring a relevant measurement range), the better compromise seemed to be a cellular density of 0.6 corresponding to an analysis duration of approximately 3 h.

3.2. Selection of a representative set of strains

The objective of this step was to constitute a set of controlled bacterial strains that were able to reliably assess the BOD_5 in a limited time and under reproducible conditions.

3.2.1. Pre-selection of bacterial candidates

The first step was dedicated to the preselection of bacterial strains that were likely to become a biological strain capable of assessing the BOD_5. A literature review identified the major phyla present in the activated sludge of wastewater treatment plants (main targets of the proposed metrological approach) (Isazadeh et al., 2016; Xia et al., 2010; Zhang et al., 2018). More than 1200 operational taxonomic units (OTUs) were identified as potential candidates in activated sludge from WWTPs. Several of these OTUs were directly rejected due to their strong pathogenicity (i.e., *Campylobacteraceae, Helicobacteraceae, Xanthomonadales,* some *Burkholderiales,* some *Bacteriodetes*, etc.) or their unsuitable metabolism (strictly anaerobic strains, such as *Desulfobacterales* and *Desulfovibrionales,* and marine strains, such as *Alteromonadales*), which were incompatible with the intended use of the technology (Fig. 3).

Among the selected families, we focused on the identification of bacterial species that met the criteria of the approach. The preselected strains should preferably be isolated from activated sludge of WWTPs, should be largely represented in these environments, should have wide and complementary *a priori* biodegradation abilities, should not be pathogenic and should be available in the current strain banks (i.e., DSMZ or ATCC). In accordance with these five criteria, 31 strains (among more than 1200 identified OTUs) were pre-selected before a second characterization step based on their ease of use (quick growth and ease of cultivation). From the obtained results, 3 strains were discarded (complexity of culture or too low a growth rate), which reduced the bacterial preselection to 28 strains (see Tables SD–1). The overall preselection strategy is shown in Fig. 3.

3.2.2. Selection of a representative set of strains

The simultaneous use of the 28 strains as individual descriptors of the BOD_5 was extremely technically complex and expensive. Consequently, we needed to reduce the number of strains used in the bioassay. First, we focused on the biodegradation capabilities of the selected strains. The biodegradation profile of each candidate was determined using 71 carbon sources (see Fig. SD-1), and hierarchical clustering was performed according to their similarities (agglomerative hierarchical clustering) (Fig. 4). Eight clusters with comparable biodegradation capabilities were identified among the 28 preselected strains (similar profiles at 95%). For example, group A, which consisted of 6 strains (*A. towneri, B. aquatica, P. pantrophus,*

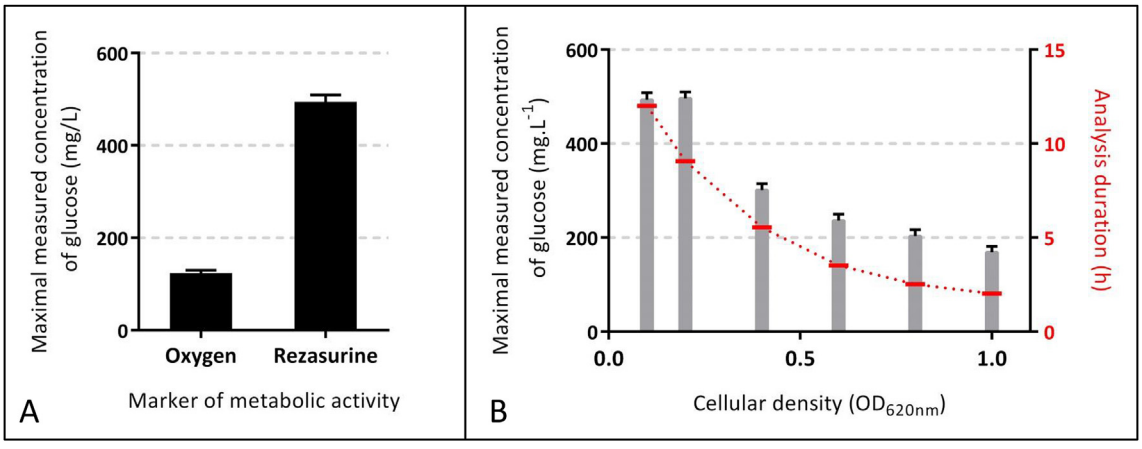

Fig. 2. Optimization of the measurement conditions. (A) Comparison of two activity marker (oxygen or resazurin) on the maximal measured concentration of glucose (strain; *E.coli*; analysis duration: 12 h; cellular density: OD_{620}nm = 0.1, standard deviation calculated from duplicates). (B) Effect of the cellular density (OD_{620}nm = 0.1/0.2/0.4/0.6/0.8/1) on the analysis duration (red dotted line) and the measurement range with resazurin (grey bars) (standard deviation calculated from duplicates). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

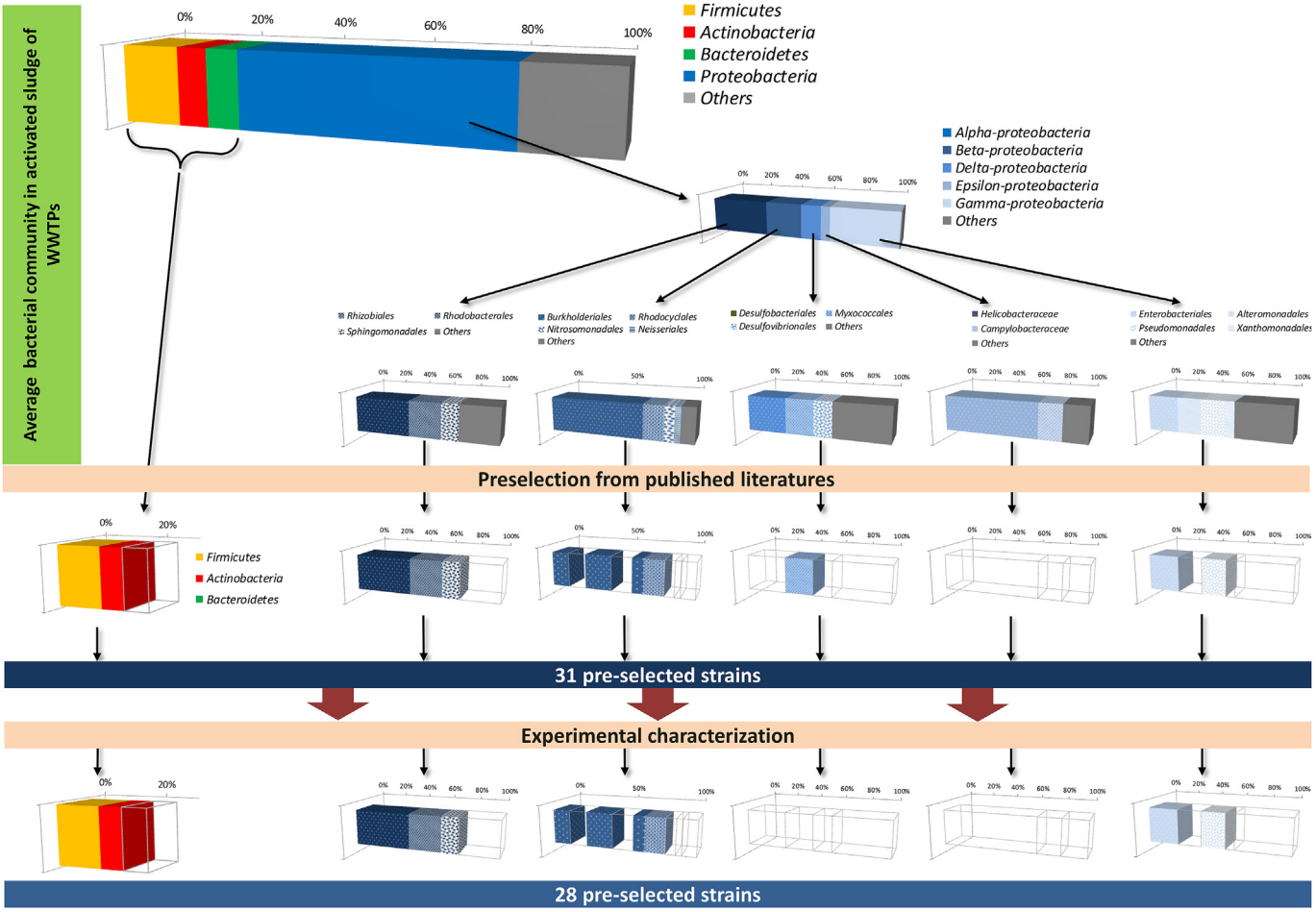

Fig. 3. Overall preselection strategy of bacterial candidates.

Z. resiniphila, B. vertrisii and *B. funiculus*), exhibited a modest range (only 6) of molecules that were likely to be biodegraded among the 71 proposed carbon sources. Conversely, group H offered a very wide set of biodegradation capabilities with ca. 85% biodegraded molecules (*R. radiobacter, B. subtilis, Staphylococcus sp., S. yanoikuyae* and *A. aurescens*).

In parallel, all strains were characterized according to their robustness and biodegradation kinetics (Fig. 5). These criteria were taken into account to select a representative among the bacterial groups with similar biodegradation profiles. To limit the effect of the water toxicity on the metrological approach, strains with strong robustness were preferred because of their inherent capabilities to resist environmental stresses. Indeed, in the case of excessive water toxicity or too high sensitivity of strains, the bacterial cells will be not able to provide reliable information inducing erroneous results. However, strains with high biodegradation kinetics were *a priori* more likely to provide a biological signal quickly. Consequently, these strains were also preferred in view of the context of technological development of this study.

Fig. 5A reports the specific robustness of each strain. These values were calculated based on 23 stress conditions and varied between 0.13 for *B. mycoides* (the most sensitive strain among the 28 tested) and 1 for *E. coli*. Fig. 5B depicts the biodegradation kinetics of each strain for the synthetic wastewater (SWW). From these data that were calculated from the maximal measured slope of oxygen consumption, we noted significant differences between strains. For example, the biodegradation kinetics reached 0.33 mg $BOD.L^{-1}.h^{-1}$ for *B. vertrisii*, whereas the kinetics achieved by *P. putida* (DSM 1868) were approximately 12 mg $BOD.L^{-1}.h^{-1}$. Consequently, the second strain should *a priori* provide a faster biological signal, as desired for our bioassay.

The final selection process of representative strains among each cluster was based on these last two criteria in addition to some criteria relative to the utilization of the strains, such as the flocculation of cells during growth (complexity of the preparation step). For example, group A consists of 6 strains (*B. funiculus, B. vertrisii, Z. resiniphila, P. pantrophus, B. aquatica* and *A. towneri*). Among these 6 strains, *B. aquatica* was selected to represent this group because of its capabilities (highest robustness and fastest biodegradation kinetics).

Finally, among the 28 candidates, a representative set of 8 strains was definitively selected, consisting of *B. aquatica, C. testosteroni, P. putida (DSM 1868), V. paradoxus, C. pseudodiphteriticum, P. mirabilis, E. coli* and *B. subtilis*.

3.3. BOD_5 assessment with the standard method and the bioassay

The BOD_5 values of the 104 real samples (collective or non-collective sanitation or stormwater) were determined according to the standard method (ISO 5815) (see Tables SD–2). As expected, the samples taken before treatment by a WWTP showed significant pollution levels, with an average BOD_5 of nearly 120 $mg.L^{-1}$ (min. 66 $mg.L^{-1}$; max. 190 $mg.L^{-1}$). The levels measured in the treated waters varied strongly according to their origins (collective or non-

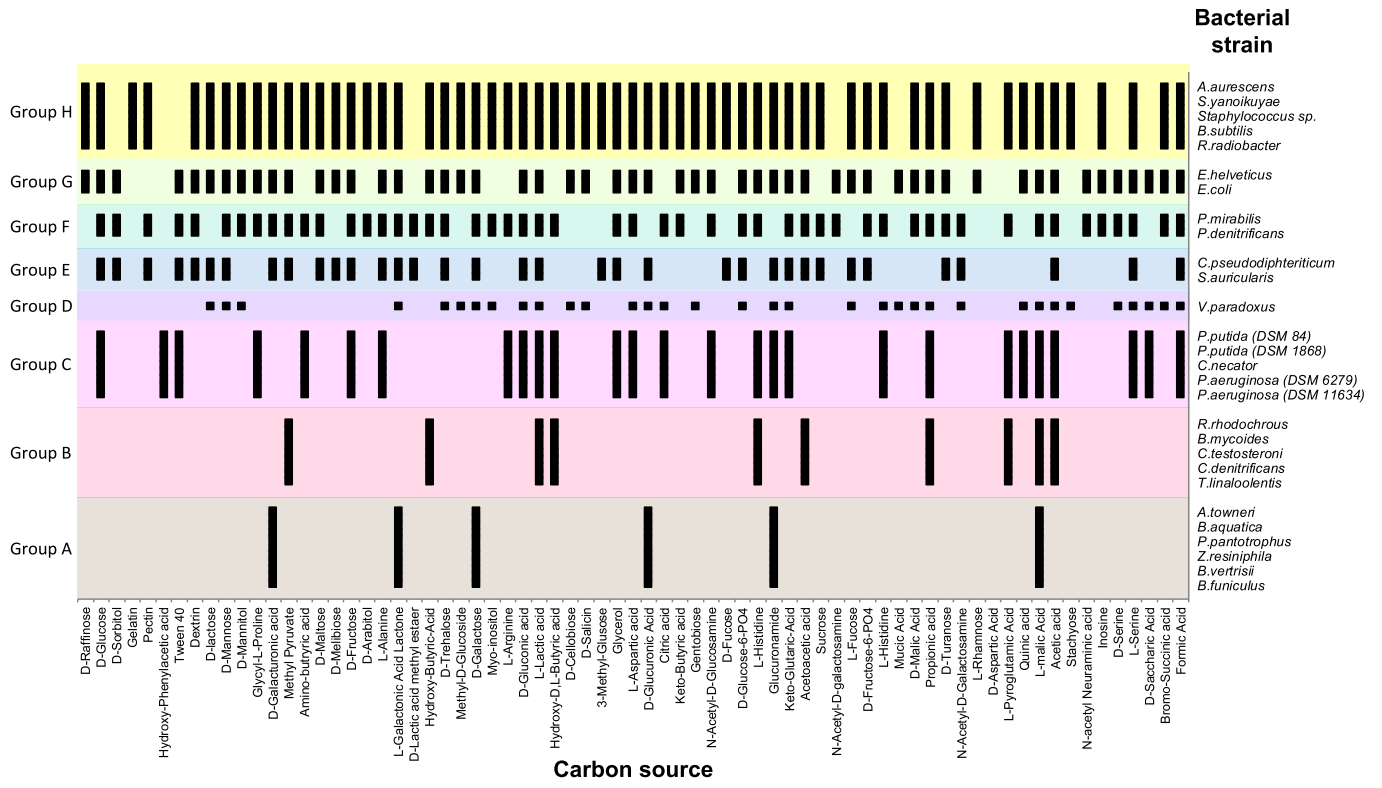

Fig. 4. Average profiles obtained after clustering of the data collected from the 28 preselected bacterial strains (clustering carried out from 71 carbon sources). Black squares correspond to the carbon sources used as substrate by the bacteria strains.

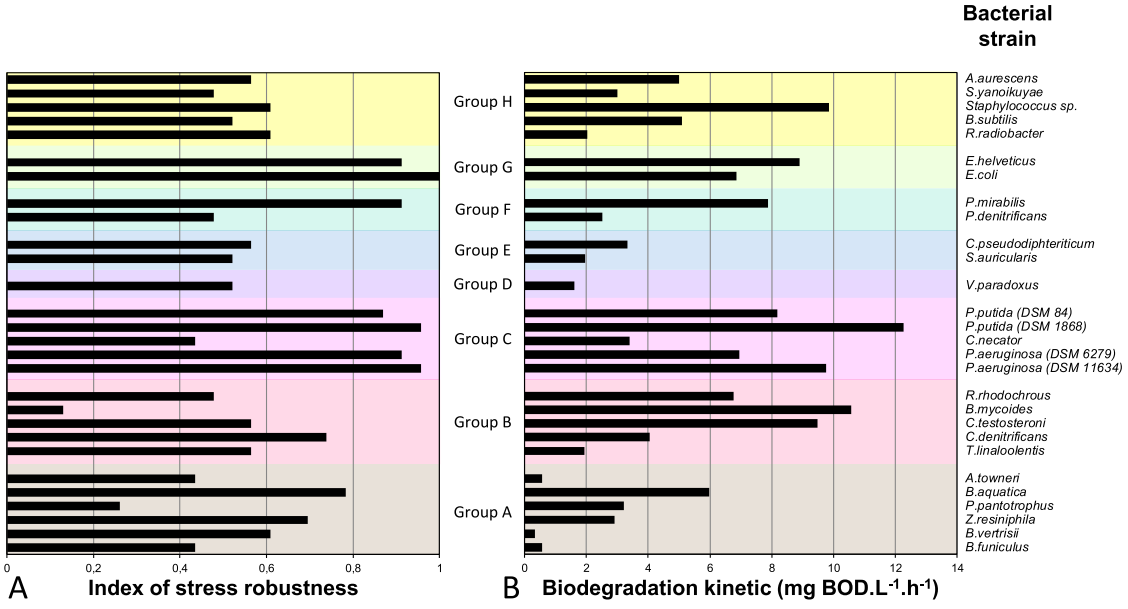

Fig. 5. Biological characterization of pre-selected candidates. (A) Index of stress robustness of strains, (B) biodegradation kinetics obtained with a standardized wastewater. For the sake of clarity, the error bars are not represented but the standard deviations do not exceed 10% between the duplicates.

collective sanitation). The average BOD_5 of stormwater was approximately 40 mg.L^{-1} (min. 12 mg.L^{-1}; max. 54 mg.L^{-1}); this pollution is probably due to runoff of water on roads and roofs, which transfers pollutants from support to water.

In parallel, each sample was subjected to the selected strains to determine the corresponding biological activity (fluorescence values obtained after 3 h of exposure for the 104 analysed samples in duplicate). The final database consisted of 208 assays. Each sample is defined by 8 fluorescence values (one per strain) and a BOD_5 reference value obtained using the standard method. The data analysis was performed from this database. The main objective of this supplementary step was to propose a reliable correlation model that allowed prediction of the BOD_5 after only 3 h. Nevertheless, we were focused on the number of strains required to

assess the BOD_5 before improving the correlation algorithm a second time (numerical model allowing calculation of the BOD_5 from fluorescence values provided by the selected strains).

3.3.1. Determination of the number of biological descriptors needed to assess the BOD_5

The data provided by each selected bacterial strain were individually correlated by linear regression with the BOD_5 reference values (Fig. 6). The average correlation rate was approximately 0.11, with a standard deviation of 0.08. Among the 8 selected strains, the best correlation rate obtained with *B. aquatica* ($r^2 = 0.2638$) was too low to be used as a reliable bio-indicator of the BOD_5.

Nevertheless, each strain provided a portion of the overall information. Consequently, increasing the number of strains used to assess the BOD_5 should improve the relevance of the approach. However, the number of strains required to ensure a reliable measurement is unknown.

Correlation models (linear regression) were designed based on the use of 1–8 strains. The obtained models are presented on Fig. 7. The correlation rate between the predicted (calculated from fluorescence data provided by the selected strains) and reference values (standard method) tended to increase with the number of strains used. Indeed, the average rate obtained from a single strain was approximately 0.11, whereas this rate reached approximately 0.6 when the 8 strains were used.

Increasing the number of strains used to assess the BOD_5 was associated with a significant increase in the correlation rate of the models. However, the predictive capabilities of the best model

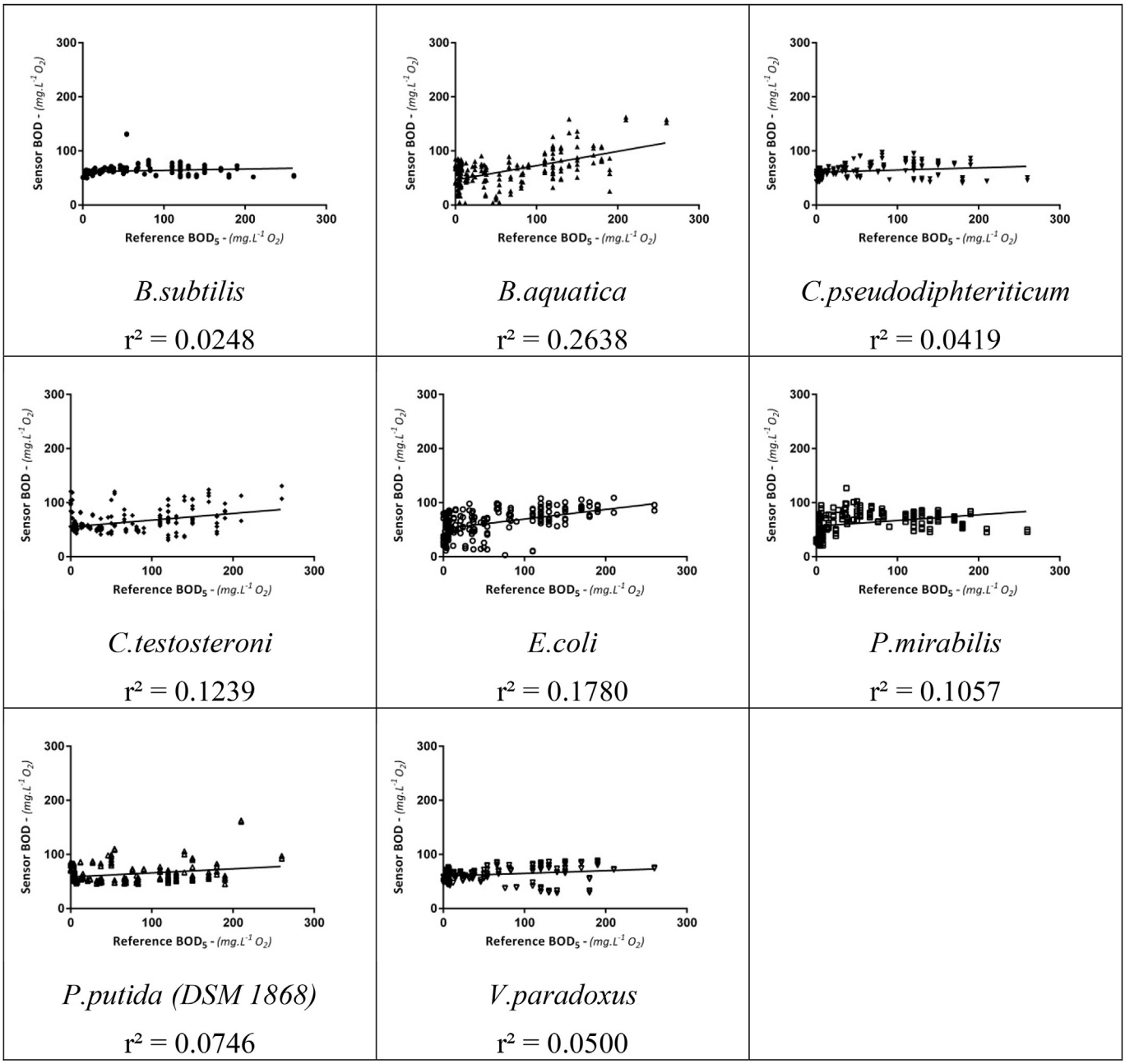

Fig. 6. Linear correlation between the fluorescence values and the corresponding reference values of BOD_5; according to an individual approach. Sensor BOD: Fluorescence value provided by the strain convert in BOD_5 equivalent.

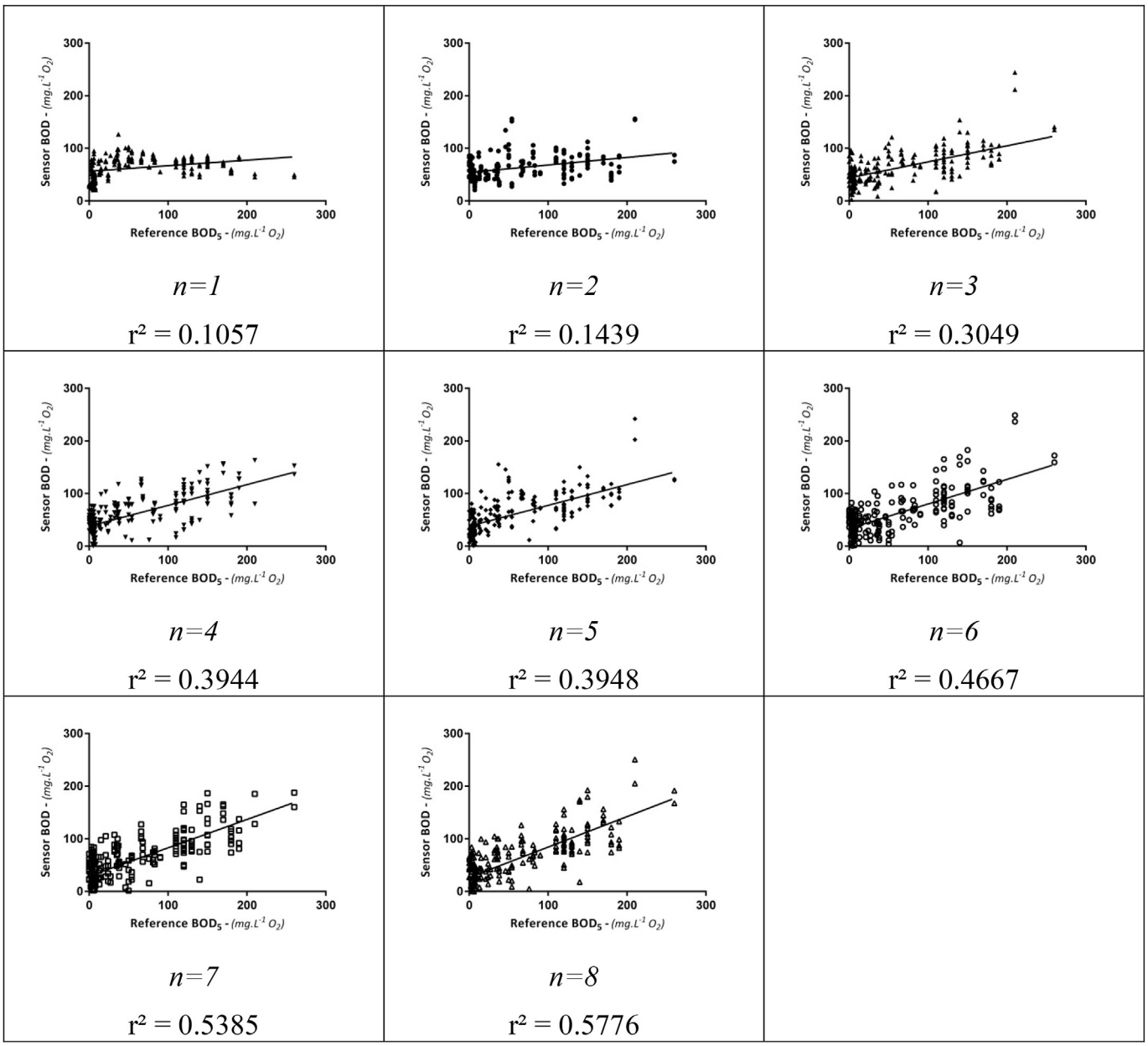

Fig. 7. Correlation rate (linear regression) obtained according to the number of used strains.Sensor BOD: BOD_5 equivalent calculated from fluorescence values of n strain(s). n = number of strains used in the linear models.

based on the 8 strains was not sufficient ($r^2 = 0.58$). In view of these results, a second step was required to improve the predictive capabilities of the data analysis model.

3.3.2. Improvement of the correlation model

To overcome this limitation and to propose a more reliable method for the assessment of the BOD_5, the regression linear algorithms were compared to a neural network approach. The neural network is a framework for many different machine learning algorithms that can process complex data. Consequently, an exploratory step was performed to screen the architectures with the most potential. Several architectures were compared with the dedicated Neuro One software (Version 6.13.0.5, Netral, France). The differences between the tested architectures were based on the number of hidden neurons, the activation mode, and the data standardization. More than 800 models (80 architectures and 10 models by architecture obtained by iterations) were generated (see Tables SD−3) by supervised learning from the overall database. The correlation rate obtained between the predicted and reference BOD_5 values varied from 0.36 (below the model based on linear regression − 0.58) to 0.93. Among the 80 tested architectures, several architectures had relevant predictive capacities with correlation rates superior to 0.8. The best model ($r^2 = 0.93$) was based on an orthonormalization (Gram Schmidt process) of inputs, 4 hidden neurons activated by a hyperbolic tangent algorithm and post-treatment of the outputs.

These models are iteratively designed from existing data (i.e., they modify the different variables that control the variables during the learning process according to the data taken into account). Consequently, over-learning is a significant risk, which leads to questions about the relevance and the robustness of the predicted BOD_5 values obtained from new data. To remove this uncertainty,

new models were designed using a fraction of the overall database.

3.3.3. Model validation

The global database was randomly separated into two sets: the learning (n = 148) and the validation (n = 60) sets (see 2.8.2). The learning set was used to design models with the 10 best *a priori* architectures determined during the previous step (see Tables SD−3, conditions overlined in grey). Ten models were generated based on the architecture. The robustness of these models was compared from unused data in the validation set based on the correlation rate between the predicted and reference values.

Among the 100 models, the most relevant was able to estimate the BOD_5 of the validation samples (data not included during the learning process) with strong reliability ($r^2 = 0.851$) (Fig. 8). This model consists of a hidden layer of three neurons activated by a hyperbolic tangent function. The input and output data are standardized with respect to a centred, reduced, normal distribution. From a global perspective, the BOD_5 values calculated from the bacterial set are relatively consistent with the reference values. The model seems to be particularly reliable between 25 and 300 mg/L^{-1} BOD_5 (according to the reference method) for wastewaters (collective or non-collective sanitation). Nevertheless, its performance decreases for samples with low biodegradable organic loads (inferior to 25 $mg.L^{-1}$ according to the reference method) and for stormwaters. Two hypotheses can explain this result: (i) the current model is not sufficiently accurate to predict low BOD_5 values (a specific model must be designed to analyse specifically these low values) and/or (ii) an organic composition of stormwater different from that of wastewaters (organic compounds collected during water runoff on roads or roofs) induces specific findings from the bacterial descriptors.

Similar results were obtained by Raud and Kikas (2013). Their strategy was based on a microbial set consisting of 7 bacterial strains selected from their intrinsic biodegradation properties. The processing of multivariate data was performed using a PLS algorithm. The validation was only performed on artificial samples (synthetic wastewater spiked with phenol, milk, fat or cellulose) and not on real wastewater samples. Nevertheless, the correlation rate between the calculated and reference values was close to 1 ($r^2 = 0.85$ in our study). Opposition to real samples seems to decrease the correlation rate (calculated values versus reference values), because mimicking a real sample with synthetic effluents is difficult.

The reference values own an inherent variability close to 20% (Jouanneau et al., 2014) notably due to the environmental variability of used inoculum. Conversely, the mean variability of data provided by the selected strains is 6.62% (results not showed). During the learning process, the model adjusts the model variables to match the data provided by the bacterial set with the reference data integrating at the same time its inherent variability (20%). To improve prediction reliability, it is required to know the "true" reference value. For this, it would be necessary to carry out several reference analysis by samples to determinate these "true" values (statistical analysis) in order to design new predictive models.

3.3.4. Post analysis

In accordance with the selected architecture, a post analysis was performed to demonstrate the relevance of the use of several strains as bio-indicators associated with data processing by the neural network. Fig. 9 shows the average correlation rates (linear least squares method) and the associated standard deviations depending on the number of strains used and according to the models used (linear regression or neural network). The results show a significant improvement in the reliability of the provided BOD_5 values compared with those obtained with the linear regression models.

With this post-analysis, we demonstrated firstly that the number of biological descriptors was a crucial factor in the development of a method to assess the biodegradable organic load. The use of a single bio-indicator seems to significantly limit the measurement performance of the strategy. The review of Jouanneau et al. (2014) tended to reinforce this observation. Indeed, among the many cited publications, strategies based on a unique biological descriptor show lower abilities to assess the BOD_5 in real samples (see Fig. SD-4) and wider variability between studies. From the publications cited in this review, the average correlation rate was close to 0.81 (SD = 0.24) with a unique biological descriptor ($r^2_{min} = 0.09$/$r^2_{max} = 1$) versus 0.95 (SD = 0.07) with a consortium ($r^2_{min} = 0.81$/$r^2_{max} = 1$). Strategies based on consortium-related systems use microbial mixtures from natural or artificial origins in most cases. These strategies are in agreement the protocol described in the reference method (ISO 5815) but induce wide inherent variability due to the descriptors used.

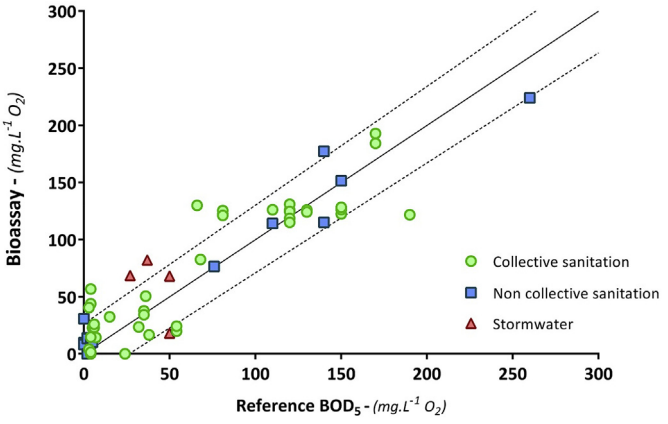

Fig. 8. Correlation between the reference values (5 days required) and the BOD_5 estimation calculated from the bacterial set (3 h required). Broken line: confidence interval of model. 1/1 correlation between calculated and reference values is shown with the center-line on the graph.

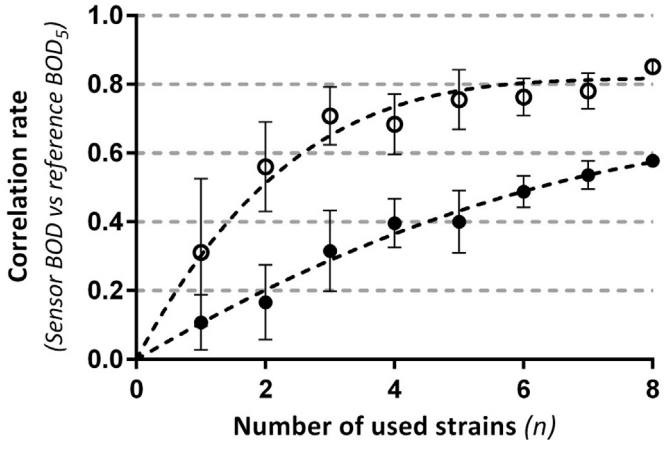

Fig. 9. Relationship between the correlation rate and the number of biological strains used to estimate the BOD_5. Correlation rates and standard deviations were calculated from available models (a single model for the overall bacterial set). Black circle: Correlation rate obtained from linear regression. Empty circle: Correlation rate calculated from neural network models.

4. Conclusion

In this work, we proposed an overall strategy to assess the BOD_5 from the selection of bacterial indicators to the validation of real wastewater samples. By acting on some levers, such as the measurement conditions or the selection of 8 bacterial bio-indicators, we developed a bioassay that allowed reliable and accurate BOD_5 measurement and required only 3 h versus 5 days for the reference method. The measurement range being enlarged, no dilution is required to assess BOD_5 in most wastewater, contrary to the reference method limited to 6 mg/L BOD. The obtained results are very encouraging and show a strong correlation with the reference BOD_5 values ($r^2 = 0.85$). Nevertheless the method seems to reach its limits regarding to the assessing of low BOD_5 levels (inferior to 25 $mg.L^{-1}$ according to the reference method). Further work will have to be carried out to improve the model in these cases.

This significant technological improvement should increase the monitoring level of wastewaters. Indeed, by reducing the analysis duration, it is easier to control the process of water treatment and, if necessary, to adjust some parameters, such as the oxygen intake or the water flow, due to the time scale that is consistent with the treatment duration (2–3 days in WWTPs).

This metrological strategy was filed with the National Institute of Industrial Property to be patented (deposit number: 1851496).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2019.115079.

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