Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Better understanding of the activated sludge process combining fluorescence-based methods and flow cytometry: A case study

Vanesa Benito [1,*], *Javier Etxebarria* [1], *Felipe Goñi-de-Cerio* [1], *Iñigo Gonzalez* [2], *Pilar Brettes* [1], *Ana Urkiaga* [1]

[1] *GAIKER, Parque Tecnológico, Ed. 202, 48170, Zamudio, Bizkaia, Spain*
[2] *EDAR Galindo, Bilbao Bizkaia Water Consortium, Sestao 48910, Bizkaia, Spain*

ARTICLE INFO

Article history:
Received 23 July 2019
Received in revised form
12 November 2019
Accepted 13 November 2019
Available online 9 December 2019

Keywords:
Activated sludge
Wastewater treatment
Cell viability
Oxidative stress
Fluorescent dyes
Flow cytometry

ABSTRACT

This study aims to demonstrate the validity of fluorescence-based methods, together with flow cytometry, as a complementary tool to conventional physicochemical analyses carried out in wastewater treatment plants (WWTPs), for the control of the currently largely unknown activated sludge process. Staining with SYTO 9, propidium iodide and 5-(and 6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) was used for cell viability and oxidative stress monitoring of the bacterial population forming the activated sludge of a WWTP. Throughout the period of research, several unstable periods were detected, where the non-viable bacteria exceeded the 75% of the total bacterial population in the activated sludge, but only in one case the cells with oxidative stress grew to 9%, exceeding the typical values of 2%–5% of this plant. These periods coincided in two cases with high values of total suspended solids (SST) and chemical oxygen demand (COD) in the effluent, and with an excess of ammonia in other case. A correlation between flow cytometric and physicochemical data was found, which enabled to clarify the possible origin of each case of instability in the biological system. This experience supports the application of bacterial fluorescence staining, together with flow cytometric analysis, as a simple, rapid and reliable tool for the control and better understanding of the bacteria dynamics in a biological wastewater treatment process.

Introduction

Since its development almost 100 years ago, the activated sludge process has been used in treating municipal and industrial wastewaters with different compositions. In this process, the organic and inorganic pollutants present in wastewater are degraded by a complex community of prokaryotic microorganisms, in which bacteria are dominant (Zhang et al., 2012; Xu et al., 2018).

Despite the importance of the biological wastewater treatment, the microbial ecology of the activated sludge process has always been a black box due to the limitations of the classical microbiological analytical methods (Douterello et al., 2014; Van Nevel et al., 2017), and the engineering perspective

* *Corresponding author.*
E-mail address: benito@gaiker.es (V. Benito).
https://doi.org/10.1016/j.jes.2019.11.012

of what the activated sludge process essentially is and how it should work (Seviour and Nielsen, 2010). Traditionally, the most widely applied techniques for monitoring and troubleshooting the activated sludge systems have been the light microscopy and the culture-dependent methods. Light microscopy allows performing morphological and some basic functional approaches, but it requires experienced technicians and does not provide direct information about physiology or activity of the biomass (Eikelboom, 2000; Spiegelman et al., 2005).

Likewise, culture-dependent approaches asses only the presence of culturable bacteria. Since a very small fraction of bacteria have been cultured so far in the laboratory (Steward, 2012; Steen et al., 2019), the non-culturable bacteria could represent an abundant or very active fraction of the total bacterial population, leading to an incomplete and mistaken view of the biological community structure (Wagner and Loy, 2002).

In order to overcome these limitations, several culture-independent techniques have been developed and applied to natural and engineered ecosystems over the last decades, such as polymerase chain reaction (PCR)-based analysis, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP) and *in situ* hybridization, providing new insights into the microbial community, its diversity, and interactions in complex environments (Gilbride et al., 2006; He et al., 2011). In addition to these, flow cytometry, combined with fluorescence dye technology, shows as a rapid, quantitative and versatile technique, that is being growingly applied to environmental microbiology (Ziglio et al., 2002; Wang et al., 2010; Ambriz-Aviña et al., 2014). It offers a realistic approach for direct and sensitive assessment of cell physiological state, activity and functionality (Saphiro, 2000; Veal et al., 2000; Czechowska et al., 2008; Díaz et al., 2010), while also allowing the detection of the whole bacterial population, including the non-culturable fraction. Actually, the results obtained from flow cytometric approaches for activated sludge studies conclude that this is a powerful tool that will enable to expand the knowledge about the dynamics of microbial communities in activated biological wastewater treatment processes (Foladori et al., 2010; Gunter et al., 2012).

One important parameter to study the dynamics of microbial populations in activated sludge is their cell viability, as they are exposed to a dynamic environment where they can experience relative fast changes (Czechowska et al., 2008). For its assessment, membrane integrity is considered one of the most conclusive criteria distinguishing between viable and dead bacterial cells (Stiefel et al., 2015; Grégori et al., 2001; Falcioni et al., 2006), as it ensures the protection of intracellular constituents and presumes the cell capability for metabolic activity, repair and reproductive growth. Cells with a permeabilized membrane cannot maintain the electrochemical gradient which generates membrane potential, and since their structures are freely exposed to the environment, they will eventually decompose, being classified as dead cells. Under these premises, viable cells are assumed to have intact and tight membranes, whereas non-viable cells have damaged or broken membranes (Nebe von Caron et al., 2000; Foladori et al., 2010).

Another interesting parameter for microbiological control is the bacterial oxidative stress, that arises when the intracellular levels of reactive oxygen species (ROS) exceed the antioxidant defenses of the cells, and lipids, proteins and DNA can be damaged (Cabiscol et al., 2000; Schieber and Chandel, 2014; Imlay, 2015). In an activated sludge process, adverse situations, such as presence of toxic compounds in the media, can lead to an elevated intracellular ROS generation, jeopardizing the bacterial well-functioning (Choi and Hu, 2008; Kamika and Tekere, 2017). Since ROS are also signaling molecules that coordinate response of bacteria under certain stress conditions (Finkel, 2011; Kashmiri and Mankar, 2014), the control of the intracellular level of ROS can allow detecting unfavorable conditions for the bacterial population before a collapse of the whole biological wastewater treatment system.

These flow cytometric analyses are carried out in combination with the use of fluorescent probes or fluorochromes. These molecules bind specifically to cell molecules or components (nucleic acids, proteins and lipids), increasing their fluorescence, or, in other cases, react with intracellular molecules to change their structure or properties, in response to the surrounding microenvironment or external changes, such as nutrient deficiency or the presence of toxic substances in the media (Díaz et al., 2010; Tracy et al., 2010).

The aim of this work was to introduce fluorescence-based techniques, combined with flow cytometry, to demonstrate their profit and reliability for monitoring the biological process of a WWTP, filling the lack of research that exists in applying these novel techniques as a monitoring tool in a real case study, in the field of the microbial ecology of the activated sludge. To meet this objective, a biological WWTP receiving municipal and industrial wastewater was chosen, in which, occasionally, instabilities occur. Cell viability and oxidative stress of the bacterial population of the activated sludge was analyzed during a one-year sampling campaign. The data collected were cross-checked with the routinely analyzed parameters of the plant, in order to establish the relationship between the state of the biological system, the operational yields of the plant, and the influence of the industrial wastewaters in the plant performance.

1. Materials and methods

The study was carried out in a WWTP located in the region of Duranguesado, in Biscay (Spain). This plant receives 65% municipal wastewater and 35% industrial wastewater from a nearby pulp and paper mill (28% of flow) and other industrial discharges, among which stand out those coming from seven zinc galvanizing companies. It performs a carbon and nitrogen removal biological process (Fig. 1), with removal efficiencies above 90% for chemical oxygen demand (COD) and above 95% for nitrogen. It has a treatment capacity equivalent to a population of 124,000 inhabitants, with a maximum treatment flow rate of 3025 m^3/hr. The activated sludge samples were collected every two weeks from October 2015 until October 2016 from aeration tanks and brought to the laboratory within one hour. More than forty physicochemical parameters, routinely analyzed in the plant, were considered during the study. In this work, only

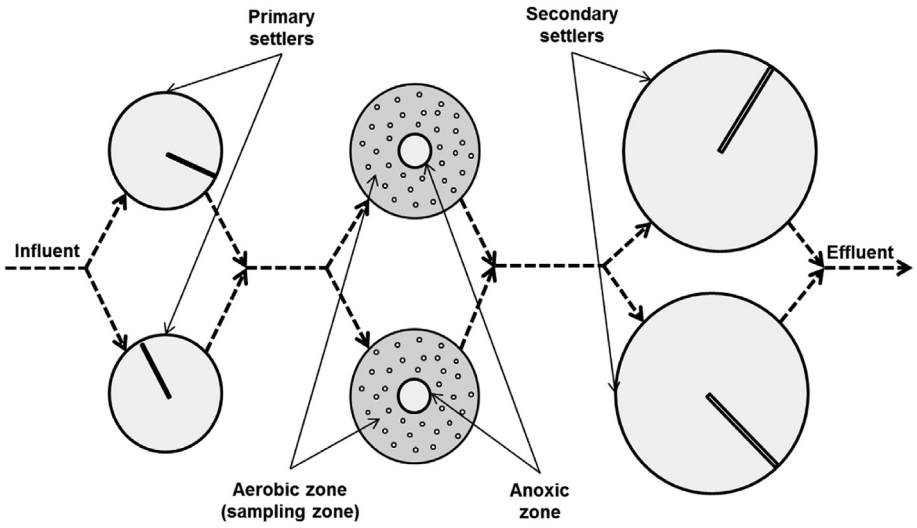

Fig. 1 – Schematic configuration of the WWTP studied. The samples were collected from the aerobic zones.

those that have been considered utterly relevant for the research have been reflected. The control chemical parameters of wastewater, as chemical oxygen demand (COD), total suspended solids (TSS), ammonia (NH_4^+-N), nitrite (NO_2^--N), zinc (Zn) and color, were measured according to Standard Methods (APHA, 2005). COD, TSS, ammonia and nitrite of the effluent were measured every day. Influent zinc was measured once every week and color of the industrial influent twice every week.

2. Fluorescence-based techniques

2.1. *Sample preparation*

The activated sludge samples were preliminary homogenized with a mechanical blender (IKA-Ultraturrax T25, IKA Labortechnik, Staufen, Germany), in order to disaggregate the activated sludge flocs and separate the single bacteria. 50 mL of the samples were subjected to three cycles of homogenization of 45 sec at 18,000 r/m. A 10–20 μm pore-size filtration was carrying out to separate coarse particles and avoid clogging of the cytometer nozzle. Finally, the DO_{600} of the bacterial suspensions obtained was adjusted to 0.2 with 1x phosphate buffered saline solution (PBS), prior to the analysis by flow cytometry.

2.2. *Cell staining*

For the cell viability analysis, bacterial suspensions were stained with the nucleic-acid binding fluorescent dyes SYTO™9 (Thermo Fisher Scientific) and propidium iodide (PI, Sigma Aldrich). Both dyes intercalate DNA but differ in their spectral characteristics and the ability of permeate bacterial membranes. Green fluorescing SYTO 9 enters all cells, viable and non-viable, while PI only permeates damaged membranes (non-viable cells), staining them red. Staining with SYTO 9 and PI was made at a final concentration of 12.5 nmol/L and 3.7 μmol/L respectively, with a 10-min incubation at room temperature in the dark.

5-(and 6)-carboxy-2′,7′-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA, Life Technologies) was used for the oxidative stress analysis. This molecule is a reduced and acetylated form of fluorescein, used as intracellular ROS indicator. This non-fluorescent molecule turns into its fluorescent form, carboxy-DFF, when, due to ROS presence, acetate groups are removed by intracellular esterases. A 4 mmol/L working solution in DMSO was prepared from the commercial original product and added to the samples at a final concentration of 10 μmol/L. Samples stained with carboxy-H_2DFFDA were incubated at 37 °C for 30 min in the dark.

2.3. *Flow cytometry*

Analysis of cell viability and oxidative stress was performed by flow cytometric measurements with a Cytomics FC500 MPL flow cytometer equipped with the SQL Server 2000 software (Beckman Coulter, USA). An excitation of 20 mW at 488 nm was used to record forward scatter, which is related to cell size, and side scatter, which is related to cell granularity/complexity. All the measurements were performed after excitation at 488 nm. SYTO 9 and carboxy-DFF fluorescence were recorded using a 525 ± 15 nm (FL1 channel, green fluorescence) band-pass filter. PI fluorescence was detected in the FL3 channel (620 ± 15 nm band-pass filter). During analytical experiments, at least 20,000 events per single determination were collected and analyzed. Constant settings of fluorescence, size and complexity parameters were controlled using calibrate beads (Beckman Coulter). Likewise, each sample was analyzed for its background autofluorescence.

3. Results and discussion

This study applied a fluorescence-based methodology, combined with flow cytometry, as a tool for monitoring the cell

viability and the oxidative stress of the bacterial population of the activated sludge, as a complement to the conventional physicochemical analysis, so as to get better understanding and control of the biological wastewater treatment process.

The cell viability and the oxidative stress of the bacteria forming the activated sludge of the WWTP were monitored every fifteen days for one year, by means of flow cytometry. Along the period of research, cell viability of the bacterial population that formed the activated sludge experienced several fluctuations, with some periods in which the viable cells outnumbered the non-viable bacteria (stable periods), and other periods where the reverse was the case, or periods of instability (Fig. 2). Among these periods of instability, three of them stood out, as the non-viable bacteria exceeded the 75% of the total bacterial population in the activated sludge. These situations took place in February, May and October 2016, and coincided with a detriment in the wastewater depuration yield in the plant. Fig. 3 shows the cytograms obtained from the flow cytometric analysis of samples stained with SYTO 9 and PI, where the differences in the fluorescent signals between a normal and an unstable situation can be seen. The oxidative stress followed a more stable behavior, with typical values of ROS positive cells between 2 and 5%, except for the sample taken on 9 February 2016, when an unusually high value of 9% was reached.

Considering the totality of results obtained along the year of study, both the cell viability and oxidative stress results, and the outputs from physicochemical measurements, it may be established that a value of $\geq$50% of viable bacterial cells, and a value below 5% of ROS positive cells, implies a normal or stable situation for this WWTP.

The attention has been focused on the critical periods when the majority of the bacterial population was damaged or died, and the good performing of the WWTP was compromised, as the depuration yields demonstrated. Among the three case studies happened in February, May and October 2016, only in one of them, that occurred in February, the nitrification process was affected, and a peak of oxidative stress was detected. On the contrary, in May and October the system maintained good ammonia removal yields, and no significant oxidative stress was observed. However, almost the entire bacterial population was damaged or dead.

In the case of February, the activated sludge sample taken on the 9th day presented a 79% of non-viable bacteria and a 9% of ROS positive cells, the highest oxidative stress value found during the research period. At that time, while no excess of COD or SST was found in the treated water effluent, there was an accumulation of ammonia and nitrite, with values of up to 2.5 mg/L of ammonia and 0.4 mg/L of nitrite, when normal values, in a situation of stability in the plant, are 0.5 mg/L and 0.1 mg/L respectively (Table 1). The analysis of metal content of the weekly composite sample of the influent wastewater carried out the week before the sampling produced Zn concentrations above the normal values (2.0 mg/L, with normal values around 0.7 mg/L).

With all the data available, it cannot be ensured the specific cause, or the set of causes, of the high percentage of non-viable cells in the activated sludge in February, the origin of the peak of the bacterial oxidative stress, and the accumulation of ammonia and nitrite in the effluent water in this month. As mentioned above, higher than normal values of zinc were detected in the industrial influent wastewater during the two weeks before the sampling. The origin of the zinc in the wastewater of this WWTP can be explained by the presence of some metallurgic industry in the vicinity, with zinc coating factories among them, whose wastewaters reach the plant as urban waters. Zinc is a heavy metal with reported toxic effect on bacteria and on activated sludge biomass, even at concentrations similar to that found in the metal analysis of the influent industrial wastewater previous to the peak of oxidative stress (Coello et al., 2002; Kelly et al., 2004; Bong et al., 2010). This metal toxicity affects also the autotrophic nitrifying community (Juliastuti et al., 2003), which some authors describe as more sensitive than heterotrophic bacteria to this kind of toxicant (Principi et al., 2006). Heavy metals like zinc act up-regulating and down-regulating several bacterial genes. One of the referenced effects of heavy metals, like zinc, on bacteria, is the up regulation of oxidative stress genes and the subsequent generation of intracellular ROS (Park and Elly, 2008; Chandran and Love, 2008). Based on these referenced facts, the presence of zinc in the influent wastewater, although surely not exclusively, could explain the peak of ROS positive cells detected by flow cytometry and the failure in the nitrification process, shown as an accumulation of ammonia and nitrite in the effluent, happened in February.

The samples collected on 20th and 24th May showed that practically the entirety of the bacterial population was damaged or died, with more than 95% of positive bacteria to propidium iodide, but without significant levels of ROS positive cells (3%–5%). During those days, there were sludge settling problems in the secondary clarifier of the plant, with the subsequent high levels of COD, up to 89 mg/L (normal value being around 45 mg/L) in the water effluent, due to an excessive presence of solids (up to 37 mg/L, when normal value was 6 mg/L). In this case, nitrification was not affected, and low levels of ammonia were found in the effluent. It was observed also an unusual dark coloring of the paper industry influent, with a high value of color (550 platinum-cobalt units, PCU), while normal values were between 100 and 150 PCU).

The situation given in October 2016 was similar to that above described for May. In the sampling carried out on 18th October, the non-viable bacteria exceeded the 90% of the total bacterial population and the ROS positive cells were a 3.5%, a normal value for this plant. Like it happened in May, unusual high levels of TSS (up to 14 mg/L, being a normal value around 6 mg/L) were detected in the treated water effluent, paper industry influent presented high color values (above 300 PCU), and no accumulation of ammonia was observed in the effluent water.

Both in May and October, the lack of significant oxidative stress and the fact that nitrification, and thus, the autotrophic nitrifying bacteria, were not affected, suggests that the presence of a toxic agent like metals was not the cause of the system instability, since it would affect both heterotrophic and autotrophic bacteria, as stated above. Then, the origin of the biological collapse should be something that affected the heterotrophic community but not the autotrophic bacteria. The main particularity that distinguishes one community from the other is the source of carbon for their growth. Heterotrophic bacteria need organic matter to synthesize vital

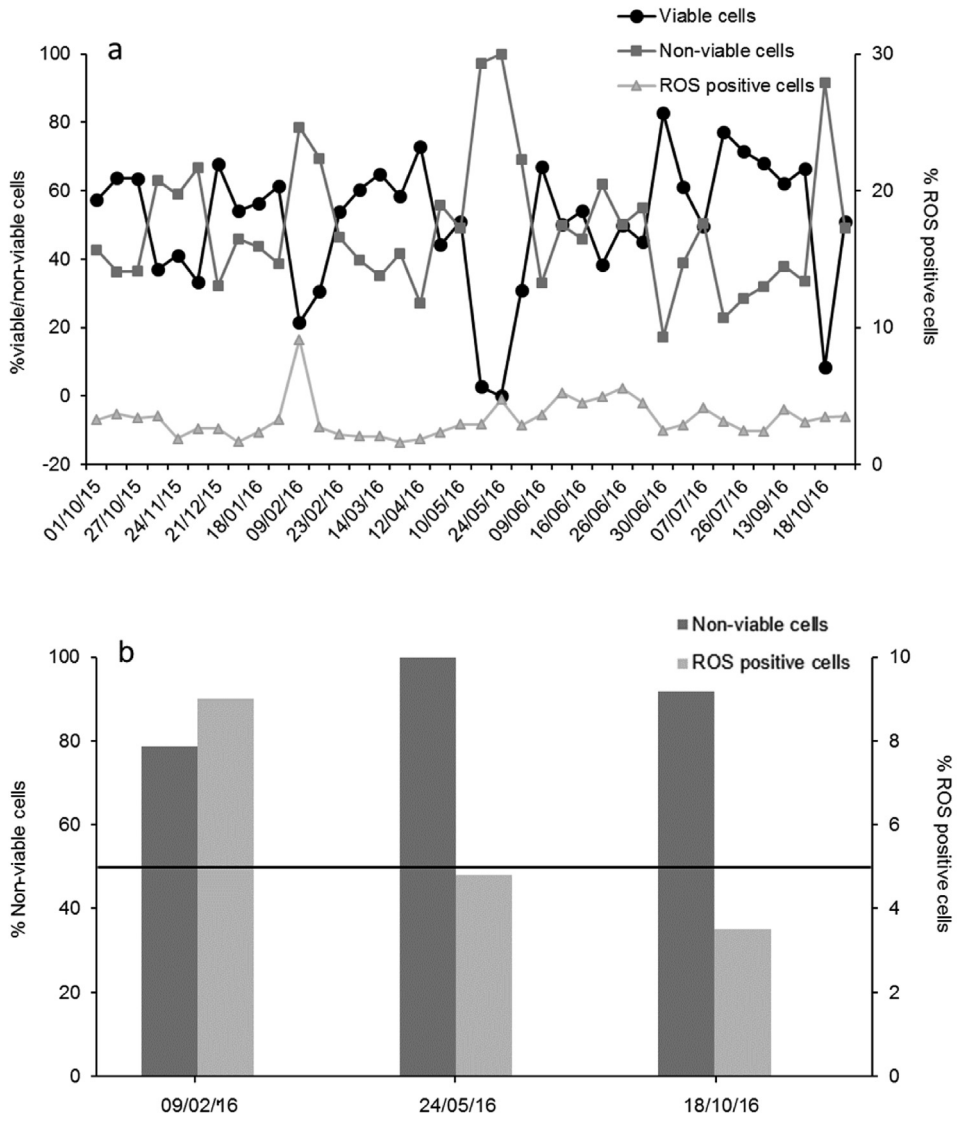

Fig. 2 – (a) Evolution of cell viability and oxidative stress of the activated sludge bacteria during the study period. (b) Detail of the situation in the unstable periods concerning the percentage of non-viable cells and cells with oxidative stress. The black line represents the normal maximum value for non-viable cells (50%) and ROS positive cells (5%) in the plant.

compounds, while autotrophic bacteria do not depend on organic matter, as they take inorganic carbon from water to transform it into complex organic molecules (Seviour and Nielsen, 2010). In a WWTP, the organic matter that feeds the heterotrophic bacteria is represented by the influent COD. Considering the biodegradable nature of the wastewaters, the total COD can be divided in biodegradable and non-biodegradable COD, being only the biodegradable fraction the one that heterotrophic bacteria degrade (Pasztor et al., 2008; Choi et al., 2017).

As it has been said above, the WWTP here studied receives the wastewaters of a neighboring pulp and paper mill industry. The composition of the wastewaters of this type of industry is conditioned by the pulp and paper-making kind of process carried out, which involve the use of diverse chemical reagents and types of raw material (wood, pulp, recycled paper) (Lacorte et al., 2003; Ashrafi et al., 2015). Some of the stages of the paper-making process provide high biochemical oxygen demand (BOD), and others recalcitrant compounds, which biodegrades reluctantly. Among the recalcitrant compounds are lignin and its derivatives, which do not provide BOD, but create strong color levels in waters (Diez et al., 2002; Selvam et al., 2011; Lindholm-Lehto et al., 2015). Thus, the appearance of dark color in the paper mill influent might indicate the presence of unusual high concentrations of lignin or lignin-derivatives compounds in the wastewater. The resulting lower biodegradability level of the wastewaters could be the reason of the massive damage or death of the heterotrophic bacteria.

Neither in May nor in October entered an unusual amount of COD in the system, but on both occasions, the effluent from paper mill industry presented high values of color. We do not

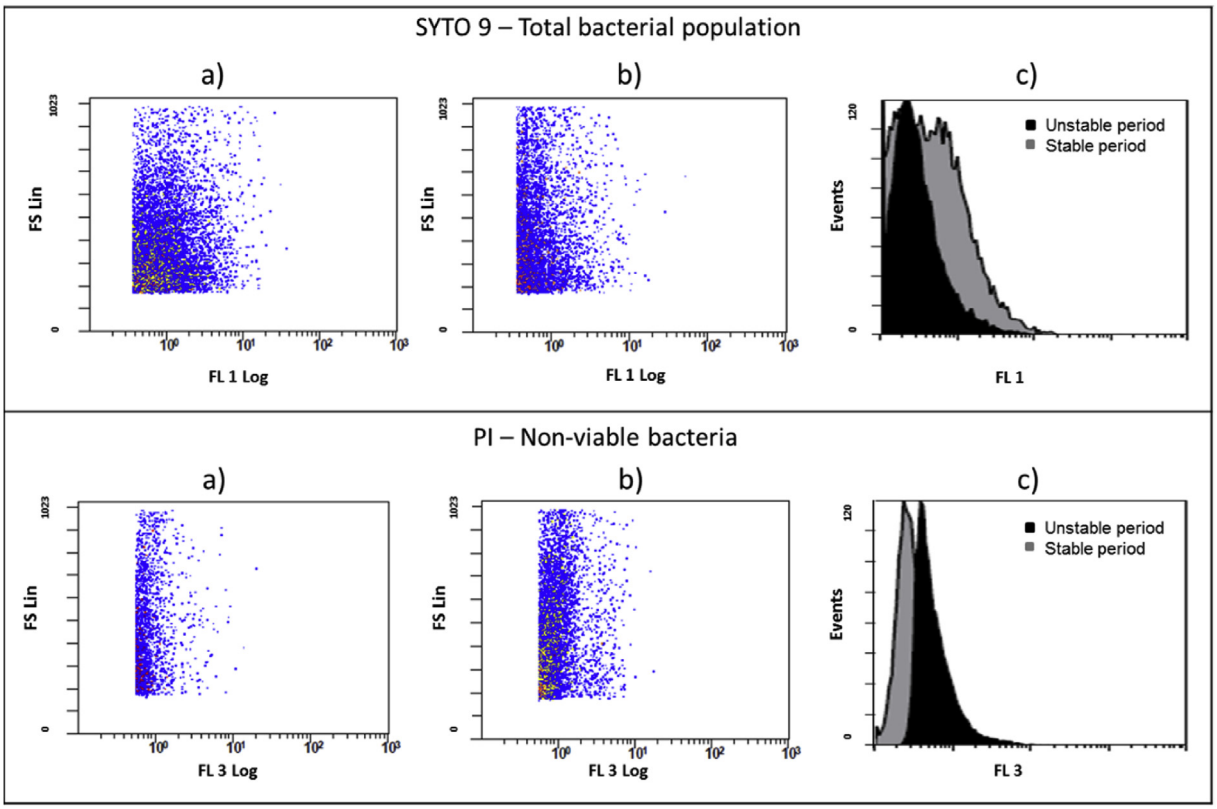

Fig. 3 – Example of flow cytometry cytograms to measure viable and non-viable bacteria, based on the staining with SYTO 9 (FL1) and PI (FL3), respectively. (a) Distribution of viable and non-viable bacteria in a stable situation; (b) distribution of viable and non-viable bacteria in a period of instability; (c) overlay fluorescence signals for stable and unstable periods.

know the biodegradability of the influent COD, because this analysis was not done at that time, but the facts here mentioned may suggest that the cause of the instability could be a change in the nature of the wastewaters from the paper mill industry, which could result in a possible change in the biodegradability of its COD that would affect negatively the heterotrophic community of the activated sludge. Either way, it would be necessary to have more data about the paper industry effluent composition in order to clarify the real causes of the situations occurred in May and October.

Table 1 – Comparison between the average data from the week before the instability periods and those in a stable situation in the studied period for influent Zn, industrial wastewater influent color and effluent COD and TSS. Figures in brackets indicate the maximum value in the week before sampling.

	February	May	October	Normal value
Influent Zn (mg/L)	2.0 (2.0)	0.9 (0.9)	1.1 (1.1)	0.7
Paper industrial influent color (PCU)	163 (190)	413 (550)	240 (310)	125
Effluent COD (mg/L)	50 (55)	70 (89)	62 (68)	45
Effluent TSS (mg/L)	6 (6)	17 (37)	12 (14)	6
Effluent NH_4^+-N (mg/L)	1.2 (2.5)	0.5 (0.5)	0.5 (0.5)	0.5
Effluent NO_2-N (mg/L)	0.4 (0.4)	0.1 (0.2)	0.1 (0.1)	0.1

4. Conclusions

Fluorescence-based techniques, combined with flow cytometry, were used for cell viability and oxidative stress monitoring of an activated sludge process. The data obtained had a clear correlation with the physicochemical parameters that conventionally have indicated the depuration yield of the system. Furthermore, the fluorescence-based techniques have shed some light on the causes of the instability situations that occasionally happened in the system, providing an information that, nowadays, operators of WWTPs cannot obtain from the traditional analytical methods. The methodology presented for monitoring the bacterial state, and the comprehensive analysis of the physicochemical and biological data, can be applied in any plant to get a total knowledge of the performance of the biological system. This methodology could provide a rapid, simple and useful tool for obtaining information about the dynamics and real state of the microbial communities in the activated sludge process, and, therefore, enhance the control of a WWTP.

Acknowledgments

This work was supported by the Bilbao Bizkaia Water Consortium.

REFERENCES

Ambriz-Aviña, V., Contreras-Garduño, J.A., Pedraza-Reyes, M., 2014. Applications of flow cytometry to characterize bacterial physiological responses. Biomed Res. Int. 2014, 461941.

APHA, 2005. Standard Methods for the Examination of Water and Wastewater. American Public Health Association.

Ashrafi, O., Yerushalmi, L., Haghighat, F., 2015. Wastewater treatment in the pulp-and-paper industry: A review of treatment processes and the associated greenhouse gas emission. J. Environ. Manage. 1 (158), 146–157.

Bong, C., Malfatti, F., Azam, F., Obayashi, Y., Suzuki, S., 2010. The effect of zinc exposure on the bacteria abundance and proteolytic activity in seawater. Interdiscip. Stud. Environ. Chem. Response. Contam. 3, 57–63.

Cabiscol, E., Tamarit, J., Ros, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int. J. Food Microbiol. 3, 3–8.

Chandran, K., Love, N.G., 2008. Physiological state, growth mode, and oxidative stress play a role in Cd (II)-mediated inhibition of Nitrosomonas europea 19718. Appl. Environ. Microb. 74 (8), 2447–2453.

Choi, O., Hu, Z., 2008. Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria. Environ. Sci. Technol. 42, 4583–4588.

Choi, Y., Baek, S., Kim, J., Choi, J., Hur, J., Lee, T., et al., 2017. Characteristics and biodegradability of wastewater organic matter in municipal wastewater treatment plants collecting domestic wastewater and industrial discharge. Water 9 (6), 409.

Coello, M.D., Sales, D., Quiroga, J.M., 2002. Toxic effects of metals on microbial activity in the activated sludge process. Chem. Biochem. Eng. Q. 16 (3), 139–144.

Czechowska, K., Johnson, D.R., van der Meer, J.R., 2008. Use of flow cytometric methods for single-cell analysis in environmental microbiology. Curr. Opin. Microbiol. 11, 205–212.

Díaz, M., Herrero, M., García, L.A., Quirós, C., 2010. Application of flow cytometry to industrial microbial bioprocesses. Biochem. Eng. J. 48, 385–407.

Diez, M.C., Castillo, G., Aguilar, L., Vidal, G., Mora, M.L., 2002. Operational factors and nutrient effects on activated sludge treatment of Pinus radiata kraft mill wastewater. Bioresour. Technol. 83, 131–138.

Douterello, I., Boxal, J.B., Deines, P., Sekar, R., Fish, K.E., Biggs, C.A., 2014. Methodological approaches for studying the microbial ecology of drinking water distribution systems. Water Res. 65, 134–156.

Eikelboom, D.H. (Ed.), 2000. Process control of activated sludge plants by microscopic investigation. IWA Publishing.

Falcioni, T., Manti, A., Boi, P., Canonico, B., Balsamo, M., Papa, S., 2006. Comparison of disruption procedures for enumeration of activated sludge floc bacteria by flow cytometry. Cytometry B Clin. Cytom. 70 (3), 149–153.

Finkel, T., 2011. Signal transduction by reactive oxygen species. J. Cell Biol. 194 (1), 7.

Foladori, P., Bruni, L., Tamburini, S., Ziglio, G., 2010. Direct quantification of bacterial biomass in influent, effluent and activated sludge of wastewater treatment plants by using flow cytometry. Water Res. 44, 3807–3818.

Gilbride, K.A., Lee, D.Y., Beaudette, L.A., 2006. Molecular techniques in wastewater: Understanding microbial communities, detecting pathogens, and real-time process control. J. Microbiol. Methods 66, 1–20.

Grégori, G., Citterio, S., Ghiani, A., Labra, M., Sgorbati, S., Brown, S., et al., 2001. Resolution of viable and membrane-compromised bacteria in freshwater and marine waters based on analytical flow cytometry and nucleic acid double staining. Appl. Environ. Microbiol. 67 (10), 4662–4670.

Gunter, S., Koch, C., Hubschmann, T., Roske, I., Muller, R.A., Bley, T., et al., 2012. Correlation of community dynamics and process parameters as a tool for the prediction of the stability of wastewater treatment. Environ. Sci. Technol. 46 (1), 84–92.

He, Z., van Nostrand, J.D., Deng, Y., Zhou, J., 2011. Development and applications of functional gene microarrays in the analysis of the functional diversity, composition, and structure of microbial communities. Front. Environ. Sci. Engin. China 5 (1), 1–20.

Imlay, J.A., 2015. Diagnosing oxidative stress in bacteria: not as easy as you might think. Curr. Opin. Microbiol. 24, 124–131.

Juliastuti, S.R., Baeyens, J., Creemers, C., Bixio, D., Lodewyckx, E., 2003. The inhibitory effects of heavy metals and organic compounds on the net maximum specific growth rate of the autotrophic biomass in activated sludge. J. Hazard. Mater. 100 (1–3), 271–283.

Kamika, I., Tekere, M., 2017. Impacts of cerium oxide nanoparticles on bacterial community in activated sludge. AMB Express 7, 63.

Kashmiri, Z.N., Mankar, S.A., 2014. Free radicals and oxidative stress in bacteria. Int. J. Curr. Microbiol. Appl. Sci. 3 (9), 34–40.

Kelly, C.J., Tumsaroj, N., Lajoie, C.A., 2004. Assessing wastewater metal toxicity with bacterial bioluminescence in a bench-scale wastewater treatment system. Water Res. 38, 423–431.

Lacorte, S., Latorre, A., Barceló, D., Rigol, A., Malmqvist, A., Welander, T., 2003. Organic compounds in paper-mill process waters and effluents. Trends Analyt. Chem. 22 (10), 725–737.

Lindholm-Lehto, P., Knuutinen, J., Ahkola, H., Herve, S., 2015. Refractory organic pollutants and toxicity in pulp and paper mill wastewaters. Environ. Sci. Pollut. Res. Int. 22 (9), 6473–6499.

Nebe-von-Caron, G., Stephens, P.J., Hewit, C.J., Powell, J.R., Badley, R.A., 2000. Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. J. Microbiol. Methods 42, 97–114.

Park, S., Elly, R.L., 2008. Genome-wide transcriptional responses of Nitrosomonas europaea to zinc. Arch. Microbiol. 189, 541–548.

Pasztor, I., Thury, P., Pulai, J., 2008. Chemical oxygen demand fractions of municipal wastewater for modelling of wastewater treatment. Int. J. Environ. Sci. Technol. 6 (1), 51–56.

Principi, P., Villa, F., Bernasconi, M., Zanardini, E., 2006. Metal toxicity in municipal wastewater activated sludge investigated by multivariate analysis and in situ hybridization. Water Res. 40, 99–106.

Saphiro, H.M., 2000. Microbial analysis at the single-cell level: tasks and techniques. J. Microbiol. Methods 42, 3–16.

Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. Curr. Biol. 24 (10), R453–R462.

Selvam, K., Priya, M.S., Sivaraj, C., 2011. Bioremediation of pulp and paper mill effluent by newly isolated wood rot fungi from western ghats area of South India. Int. J. Pharm. Biol. Arch. 2 (6), 1765–1771.

Seviour, R., Nielsen, H. (Eds.), 2010. Microbial Ecology of Activated Sludge. IWA Publishing.

Spiegelman, D., Whissel, G., Greer, C.W., 2005. A survey of the methods for the characterization of microbial consortia and communities. Can. J. Microbiol. 51, 355–386.

Steen, A.D., Crits-Christoph, A., Carini, P., DeAngelis, K.M., Fierer, N., Lloyd, K.G., et al., 2019. High proportions of bacteria and archaea across most biomes remain uncultured. ISME J. 13, 2125–2128.

Steward, E.J., 2012. Growing Unculturable Bacteria. J. Bacteriol. 194 (16), 4151–4160.

Stiefel, P., Schmidt-Emrich, S., Maniura-Weber, K., Ren, Q., 2015. Critical aspects of using bacterial cell viability assays with the fluorophores SYTO 9 and propidium iodide. BMC Microbiol. 15, 36.

Tracy, B.P., Gaida, S.M., Papoutsakis, E.T., 2010. Flow cytometry for bacteria: enabling metabolic engineering, synthetic biology and the elucidation of complex phenotypes. Curr. Opin. Biotechnol. 21, 85–99.

Van Nevel, S., Koetzsch, S., Proctor, C.R., Besmer, M.D., Prest, E.I., Vrouwenvelder, J.S., et al., 2017. Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. Water Res. 113, 191–206.

Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunol. Methods 243, 191–210.

Wagner, M., Loy, A., 2002. Bacterial community composition and function in sewage treatment systems. Curr. Opin. Biotechnol. 13, 218–227.

Wang, Y., Hammes, F., De Roy, K., Verstraete, W., Boon, N., 2010. Past, present and future applications of flow cytometry in aquatic microbiology. Trends Biotechnol. 28 (8), 416–424.

Xu, S., Yao, J., Ainiwaer, M., Hong, Y., Zhang, Y., 2018. Analysis of Bacterial Community Structure of Activated Sludge from Wastewater Treatment Plants in Winter. Biomed Res. Int. 2018, 8278970.

Zhang, T., Shao, M., Ye, L., 2012. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J. 6, 1137–1147.

Ziglio, G., Andreottola, G., Barbesti, S., Boschetti, G., Bruni, L., Foladori, P., et al., 2002. Assessment of activated sludge viability with flow cytometry. Water Res. 36, 460–468.