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Flow cytometry, a sustainable method for the identification and quantification of microorganisms in enology - Part 2/2 Practical and environmental benefits of flow cytometry applied to wine microbiology

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Flow cytometry (FC) coupled with the use of different markers represents an analytical tool that is fully in line with the wine sector's requirements. This article presents the various applications of this technique in wine microbiology. Apart from its technical benefits, FC is in tune with the commitment to sustainable development, a major issue for the sector.

Monitoring fermentation activity

FC is ideally suited to the quality control of a starter culture, providing quick quantitative and qualitative analysis of the population. Results are obtained in less than half a day, allowing the winemaker to quickly decide whether or not to use the starter. In the event of a sluggish alcoholic fermentation (AF) or malolactic fermentation (MLF), rapid determination of the percentage mortality of the microbial population can guide the winemaker's choices.

Avoiding spoilage by *B. bruxellensis* during aging

To avoid spoilage due to the growth of *B. bruxellensis*, we have developed a two-step surveillance strategy. The first step consists in searching for the presence of active yeasts. If no active yeast is detected, there is no need to search for *B. bruxellensis*. If active yeasts are detected, on the other hand, specific markers are used. In addition to species-specific quantification and the provision of essential information on the physiological state of the population, this approach repositions *B. bruxellensis* in the overall microbiological context of the sample.

Phenotyping *B. bruxellensis* isolates

For phenotyping isolates, a study was conducted using 24 *B. bruxellensis* colonies isolated from wines from 10 Bordeaux vineyards in the 2020 vintage. This study was carried out using a culture medium and under standardized conditions (Figure 1, supplementary data). This work demonstrated disparate behavior regarding the rate of volatile phenol production. Parallel monitoring of the *B. bruxellensis* population using specific FC confirmed that the isolates that were quickest to produce volatile phenols had a higher growth rate and thus created a large volume of biomass more rapidly[1]. This universal capacity of the species for spoilage and its disparities in the rate of volatile phenol production had already been observed by Cibrario *et al.* (2019)[2]. These observations suggest that certain strains have a higher spoilage potential than others.

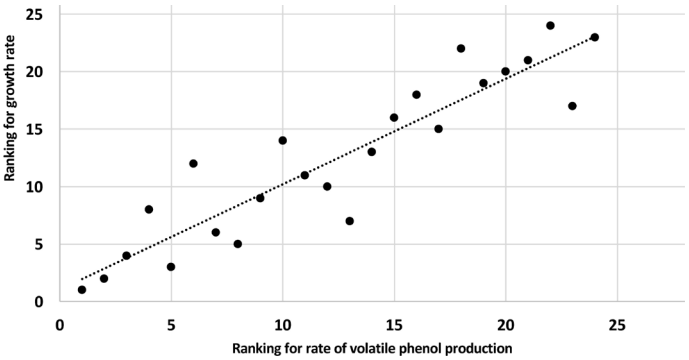

FIGURE 1. Comparison of 24 strains isolated from 10 Bordeaux vineyards. Correlation between volatile phenol production and rapidity of growth on culture medium.
Rankings were determined on day 4, at the end of the exponential phase / start of the stationary phase. The initial *B. bruxellensis* population was 102 cells/mL.

Quality control of cleaning/disinfection protocols for cooperage oak

FC is a valuable tool for monitoring wooden vessels. It is now used routinely to validate or optimize cleaning/disinfection protocols through the use of a special tool to sample wood inside the vessel[3].

Quality control of packaging operations

FC allows for fast and efficient quality control of packaging operations. The speed of analysis allows implementation of direct corrective measures, e.g. in the event of loss of filter integrity. To check compliance with certain technical specifications, specific protocols can be applied to reduce the detection threshold to 0.5 cell/mL on a wine matrix.

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Environmental benefits of FC* compared with alternative techniques for the quantification of *B. bruxellensis* (*study valid for our total yeasts and specific FC method)

Environmental impact assessment of the analytical procedures was conducted based on a new tool described by Płotka-Wasylka in 2018[4], the Green Analytical Procedure Index (GAPI), which differs from a life cycle analysis. It provides an environmental impact assessment of the entire analytical process, based on the classification criteria shown in Table 1. GAPI analysis results in a graphic representation using a color code (green/yellow/red). As shown in Figure 2, each section of the various pentagons represents one aspect of the analytical procedure. The green color represents those with the lowest environmental impact.

TABLE 1. Description of GAPI index parameters (after Płotka-Wasylka, 2018[4]).

Category	Green	Yellow	Red
Sampling / Sample transport			
Collection (1)	Real-time measurement on site / Instrument permanently on site.	Sampling then measurement on site / Instrument taken on site as needed.	Sampling on site and analysis in the laboratory.
Preservation for transport (2)	None	Chemical or Physical	Physico-chemical
Transport (3)	None		Required
Storage (4)	None	Normal conditions	Special conditions
Type of method			
Direct or indirect (5)	Ø sample preparation	Simple procedures (e.g. filtration, decanting)	Extraction required
Central circle in 5: Qualification and quantification procedure		No central circle in 5: Qualification procedure only	
Sample preparation			
Scale of extraction (6)	Nano-extraction	Micro-extraction	Macro-extraction
Solvents/reagents (7)	Ø solvents	"Green" solvents/reagents	"Non-green" solvents/reagents
Additional treatment (8)	None	Simple treatment (clean-up, solvent removal)	More advanced treatment (e.g. derivatization, mineralization, etc.)
Reagents and solvents			
Quantity (9)	<10 mL (<10 g)	10-100 mL (g)	>100 mL (g)
Health risk (10)	Slightly toxic, slight irritant; NFPA* health-hazard score = 0 or 1	Moderately toxic, can cause temporary incapacity; NFPA = 2 or 3	Severe injury from short-term exposure; known or suspected small-animal carcinogen, NFPA = 4
Safety risk (11)	NFPA flammability or instability score = 0 or 1 No specific hazard	NFPA flammability or instability score = 2 or 3 or presence of a specific hazard	NFPA flammability or instability score = 4
Instrumentation			
Energy (12)	≤0.1 kWh per sample	≤1.5 kWh per sample	>1.5 kWh per sample
Occupational risk (13)	No emission	-	Emission of vapors to the atmosphere
Waste (14)	<1 mL // <1 g per sample	1-10 mL // 1-10 g per sample	>10 mL // >10 g per sample
Waste treatment (15)	Recycling	Degradation, passivation	No treatment

*NFPA: National Fire Protection Association.

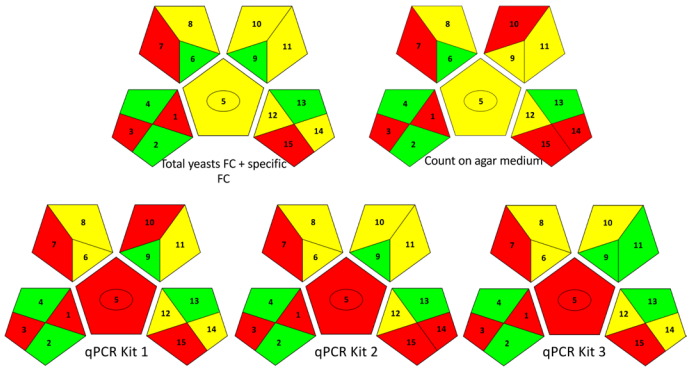

FIGURE 2. GAPI pentagons for the main methods used for *B. bruxellensis* quantification. The numbers in the pentagons refer to the different steps in the GAPI index in Table 1.

Our method of quantifying *B. bruxellensis* yeasts by FC (total yeasts and species-specific quantification of *B. bruxellensis)* was assessed with this tool and compared with other routinely used techniques: culture on nutrient agar medium and quantitative PCR (qPCR). For qPCR, analysis was performed using the three most commonly used commercial kits based on available data (analysis protocols and safety data sheets).
The GAPI analysis (Figure 2) illustrates the advantages of FC compared with its alternatives for various sustainable production criteria.

Reagents

FC analysis is a direct method, avoiding the need for any dilution or extraction during sample analysis, which is not the case for qPCR (see sections 5 and 6 and Figure 2).
FC coupled with a viability marker or with our species-specific *B. bruxellensis* marker does not require the use of chemicals that are hazardous to health, as is the case for culture on nutrient agar medium (antibiotics) and qPCR (preservatives used in some kits) (see section 10 in Figure 2).

Energy consumption and waste production (Figure 3)

Concerning Petri dishes (3 dishes / sample = 3 dilutions), this method consumes half as much energy as FC. However, it generates 17 times the amount of plastic waste, making it by far the least sustainable method in this respect.
Regarding qPCR methods, they consume 21 to 36 % more energy than FC. Considering the annual volume of samples processed, these differences have a real impact on the overall consumption of the laboratory. Regarding the generation of plastic waste, the differences are even more marked, with qPCR kits generating twice as much as FC on average.

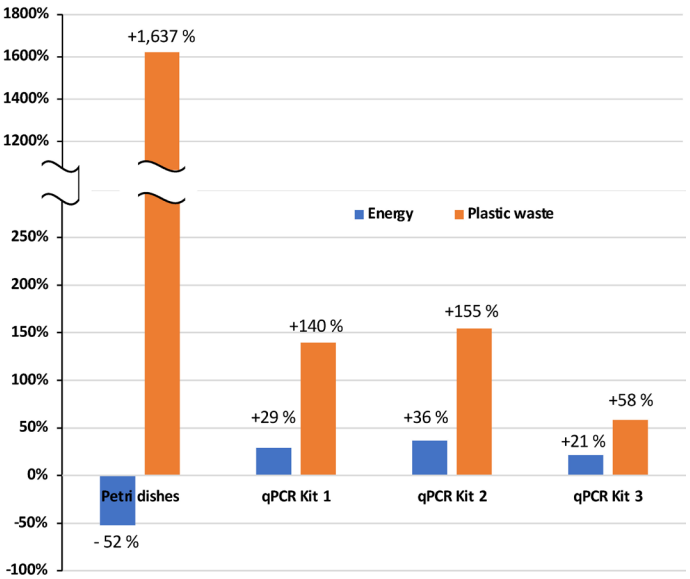

FIGURE 3. Energy consumption and quantity of waste generated per sample compared with our total yeasts FC and specific FC method.

Conclusions

FC is a real asset in the field of rapid diagnostics in enology. Its reliability, short turnaround time, low cost and the level of information provided on the microbial population (species-specific quantification, physiological state and overall microbiological context) make it a tool of choice for checks in the field. Despite its apparent ease of use, it nevertheless requires a high level of expertise to interpret the results and to develop labeling methods.
In light of the GAPI analysis, and all the benefits presented, FC is a method that offers a higher level of information for a lower environmental impact than currently available alternatives. ■

1 Chandra, M., Madeira, I., Coutinho, A.-R., Albergaria, H., & Malfeito-Ferreira, M. (2016). Growth and volatile phenol production by Brettanomyces bruxellensis in different grapevine varieties during fermentation and in finished wine. *European Food Research and Technology*, 242(4), 487-494. https://doi.org/10.1007/s00217-015-2559-y

2 Cibrario, A., Miot-Sertier, C., Paulin, M., Bullier, B., Riquier, L., Perello, M. C., de Revel, G., Albertin, W., Masneuf-Pomarède, I., Ballestra, P., & Dols-Lafargue, M. (2019). Brettanomyces bruxellensis phenotypic diversity, tolerance to wine stress and wine spoilage ability. *Food Microbiology*, 103379. https://doi.org/10.1016/j.fm.2019.103379

3 David, V., & Alexandre, H. (2019). Le contrôle microbiologie des fûts et barriques, La Revue des Œnologues | Revue des Œnologues n°173. *La Revue des Œnologues*. https://search.oeno.tm.fr/sommaires

4 Płotka-Wasylka, J. (2018). A new tool for the evaluation of the analytical procedure : Green Analytical Procedure Index. *Talanta*, 181, 204-209. https://doi.org/10.1016/j.talanta.2018.01.013