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Short statistical summary

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ABSTRACT

The current experimental study investigates the influence of latex microsphere particles' size on the assessment of their hydrophilic/hydrophobic character, using the method known as “Microbial Adhesion to Hydrocarbons” (MATH). Since bacteria surfaces often change according to the environment in which they find themselves, most of the experiments here were carried out using the calibrated latex microspheres Polybeads® and Yellow-green Fluoresbrite® (Polyscience) microspheres with diameters between 0.2 µm and 4.5 µm. All the beads had a density of ~1.05 g/cm^3. The first set of experiments was performed to adapt the procedure for measurements of water contact angles to microsphere lawns. It was found that all the microspheres tested were hydrophobic, when using a water contact angle of around 110-118°. However, wide differences were observed using the MATH method. The smaller microspheres (0.2 µm, 0.5 µm +/- 0.75 µm) exhibited a poor affinity to hexadecane, even after long contact times, suggesting a hydrophilic character. In contrast, larger microspheres quickly adhered to hexadecane, which is consistent with the values obtained for the water contact angles observed. These results suggest that, at least where hydrophobic particles are concerned, the MATH method is not suitable for the assessment of the hydrophobic character of particles with diameters of less than 1.0 µm. We lastly investigated whether the data obtained for *Bacillus* spores could also be affected by spore size. The hydrophobicity of spores of eight *Bacillus* strains was analysed by both MATH and contact angle. Some discrepancies were observed between both methods but could not be related their size (length or width).

Keywords: *Bacillus* spores, particle size, microspheres, hydrophobicity, MATH, water contact angle

1. Introduction

In the environment, bacteria can be seen to be adsorbed at air/liquid, air/solid, or at liquid/liquid interfaces. Once adsorbed, some bacteria continue to grow and if environmental conditions allow, these adherent bacteria will produce complex structures called “biofilms”. Unfortunately, in many instances, biofilms cause serious damage and disease, such as in medical environments [1]. This is also the case in the food industry. Here, contamination of food processing lines surfaces and equipment by pathogens and spoilage bacteria is a major issue [2] that has yet to find a proper cleaning and disinfection solution. Indeed, after hygienic procedures, adherent bacteria are still commonly found on surfaces, mostly in the form of adherent spores, e.g. *Bacillus* spores, but also in the form of biofilms [3]. These contaminated surfaces seem to be the cause of food re-contamination which could give rise to serious economic and health problems [4]. Consequently, microbiological contamination costs the food industry several million dollars annually [5].

Despite the widely varying properties of food-industry plant surfaces, both adherent bacteria and biofilms can be observed on all of them, from stainless steel devices and flooring to belts or rubber seals [6,7]. Hydrophobicity is suspected of being a significant property in determining the strength of the interaction between bacteria or biofilms and surfaces with which they come into contact [8]. Various works have suggested that the hydrophobicity of *Bacillus* spores increases their capability of contaminating stainless steel. One study of *B. subtilis* spores demonstrated how their highly hydrophilic character was due to the mucous layer surrounding spores. Indeed, the mechanical removal of this layer made the spore hydrophobic and the number of bacteria which could contaminate stainless steel surfaces increased more than tenfold [9]. Another study involved the analysis of a panel of 17 spores belonging to 7 *Bacillus* species [10]. While the strongly hydrophilic B. subtilis spores were poorly adherent to stainless steel (around 10^3 spores cm^{-2}), high *B. cereus* contamination levels (up to $2x10^5$ spores cm^{-2}) were observed, the spores of which are known to be hydrophobic to highly hydrophobic. Similar results have been reported for a panel of 16 bacterial

70 strains including known pathogens [11]. On analysis of the hydrophilic/hydrophobic properties of the
71 bacteria and their ability to contaminate polystyrene disks (% coverage), the authors found coverage
72 values below 50% for hydrophilic bacteria, yet over 80% for hydrophobic bacteria.

73 Therefore, in order to assess a given bacteria's predisposition for adhesion and ease of cleaning,
74 appropriate methodologies are crucial for estimating this bacteria's hydrophobicity. There are
75 several well-developed conventional methods for evaluating bacterial hydrophobicity, each with its
76 own advantages and disadvantages. These include two-phase partitioning systems, hydrophobic
77 interaction chromatography or the measurement of water contact angles. Conventionally, the water
78 contact angle (CA) is used to evaluate the hydrophilic/hydrophobic character of solid surfaces. Solid
79 surfaces with CAs greater than 90° are traditionally considered to be hydrophobic, notably based on
80 the adhesion interaction between water and materials. However, other studies have concluded that
81 the threshold value between hydrophilic and hydrophobic materials lies at 65°, because the scraping
82 of materials results in an increase in the CA for initial values over 65° and a decrease for initial values
83 lower than 65° [12]. Several methods have been developed (static vs dynamic CA measurement, air
84 vs liquid environment) and for each method, numerous operational parameters vary, depending on
85 the research team (use of an equilibration step, time and length of the drying step…). The main
86 drawbacks of these methods is that they require a preliminary step for the production of the
87 bacterial lawns on which water droplets will be placed and that CA measurement on bacterial lawns
88 strongly depend on environmental conditions [13].

89 A quick, easy and commonly-used experimental technique to estimate particles or spores'
90 hydrophilicity or hydrophobicity is the "Microbial Adhesion to Hydrocarbons" (MATH) method. This
91 consists of a differential partitioning of bacteria at a hydrocarbon/aqueous interface. In short, the
92 method evaluates the decrease in the particle concentration in an aqueous suspension, by measuring
93 its absorbance before and after vortexing the particle suspension with a hydrocarbon. Indeed, the
94 stronger the hydrophobicity, the greater the adhesion to the interface and consequently the sharper
95 the absorbance decrease. Hexadecane is probably the most frequently employed apolar solvent,

because it is considered to be the most efficient at partitioning, although toluene is more effective when spores are analysed [14]. As MATH is such a simple and fast method of hydrophobicity assessment, requiring only a little inexpensive laboratory equipment, such as a vortex mixer and a spectrophotometer, it is used in a wide variety of areas, from the food environment [15] to environmental engineering [16]. Though very popular, this assay still suffers from a significant deficit in standardisation. However, previous studies have investigated the effects of variations in some of the operating parameters like vortex duration, hydrocarbon selection and hydrocarbon-aqueous phase volume ratio [17,18]. Indeed, these studies have highlighted, in particular, that the hydrophobic or hydrophilic classification of some bacteria would depend on the vortex duration or on the hydrocarbon volume employed. Surprisingly, little or no investigation has been initiated into the potential impact of bacterial properties, including their size on the evaluation of their hydrophobicity.

In order to investigate this possible impact of particle size, we first analysed microspheres of different sizes using MATH and goniometry. We then analysed *Bacillus* spores properties using these two methods and compared the data obtained.

2. Material and Methods

2.1. Polystyrene microspheres and Bacillus *spores*

A set of experiments was performed with two series of monodisperse polystyrene (latex) microspheres of different sizes (Polysciences Inc), close to those of bacterial spores : Polybeads® microspheres (0.2 µm, 0.5 µm, 0.75 µm, 1.0 µm, 2.0 µm, 3.0 µm, and 4.5 µm in diameter) and Fluoresbrite® Dyed Yellow-Green (YG) microspheres (0.2 µm, 0.5 µm, 0.75 µm, 1.0 µm, 2.0 µm, and 3.0 µm in diameter, the 4.5-µm Fluoresbrite microspheres not being commercially available). All bead densities were around 1.05 g/cm^3. According to the manufacturer, the fluorescent

microspheres were internally dyed using solvent swelling / dye entrapment, which meant the highly hydrophobic yellow-green dye would remain trapped in the beads in aqueous environments. The microspheres are classified as being hydrophobic with a slight anionic charge.

We first used Fourier Transform Infrared (FTIR) spectrometry to check that the both kinds (YG and Polybeads) of microsphere composition was similar independently of their size. The FTIR spectra of dried microspheres were recorded with a FTIR spectrometer (Tensor 37, Brucker) using a diamond ATR accessory. All the spectra (Figure 1) were acquired between 4000 and 400 cm^{-1} with 32 accumulations and a spectral resolution of 4 cm^{-1}. Water vapor subtraction and baseline correction were performed. The spectra recordings and data were processed using Bruker OPUS 7.5 software. Similar patterns were obtained, thereby suggesting all the microspheres tested, shared a fairly uniform composition.

Other experiments were performed with *Bacillus* spores of different sizes and hydrophobic properties. Six of the strains tested belonged to the *B. cereus* group (with an exosporium): *B. cereus* ATCC 14579 (Bc 14579), CUETM 98/4 (Bc 98/4), and D6 (Bc D6), *B. thuringiensis* 407cry- (Bt 407) and LMG 7138 (Bt 7138), *B. anthracis* 9131 (Ba 9131) [19], lacking both pXO1 and pXO2 plasmids. Two strains belonging to other *Bacillus* species were also analysed: *B. subtilis* LMG 7135 (Bs 7135) and *B. pumilus* 98/7 (Bp 98/6). Spores were produced on Spo8-agar at 30°C. Spo8-agar consisted (per litre) of 8 g nutrient broth (Biokar Diagnostics, Beauvais, France) and 15 g agar, complemented with 0.51 g $MgSO_4.7H_2O_2$, 0.97 g KCl, 0.2 g $CaCl_2.2H_2O$, 3.10^{-3} g $MnCl_2.4H_2O$, $0.55.10^{-3}$ g $FeSO_4.7H_2O_2$. When over 95% of spores were obtained, they were harvested by scraping the surface, washed five times in sterile water and stored in sterile water at 4°C until use. Before each experiment, two further washes were performed.

Before analysis, spore and microsphere suspensions were subjected to a 2.5-min ultrasonication step in an ultrasonic cleaner (Bransonic 2510E-MT, 42 kHz, 100 W, Branson Ultrasonics Corporation, USA) to limit the presence of aggregates. Indeed, the presence and the size of the spore aggregates

147 varied considerably during storage at 4°C and may reach hundreds of spores for some *B. cereus*
148 species, probably due to their hydrophobic character, as previously shown on *Propionibacteria* [20].
149
150 *2.2. Contact angle measurement*
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152 An extensive method development phase was required before undertaking the measurements of
153 contact angles on the different particles. The experimental method developed is as follows. Spores or
154 microspheres were collected by filtration through 0.22 µm cellulose ester (VSWP02500, Millipore)
155 filters in order to obtain a regular/flat layer of spores or microspheres (Figure 2A). Filters were then
156 fixed on glass slides and left to air-dry at 20°C for 2 h. The glass slides with filters were mounted on
157 the contact angle-measuring instrument table (Digidrop, GBX, France). A drop of water (5 µl) was
158 placed on the particle lawn and an image of the droplet was captured (after 320 ms in most cases or
159 after 1 s for some spore lawns, to allow the droplet to become steady), from which a contact angle
160 measurement was obtained. In this study, a particle was considered to be hydrophobic for water
161 contact angles > 90°, hydrophilic for lower values.
162
163 *2.3. Affinity to hexadecane*
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165 The partitioning method based on the affinity of spores to an apolar solvent, hexadecane (Sigma)
166 was used with some modifications. Spores and microspheres were re-suspended in saline, the
167 suspensions being at an absorbance of 0.5 to 0.6 at 600 nm (A_0) in glass tubes (10 mm in diameter ×
168 75 mm). Three milliliter aliquots of the suspensions and 500 µL of hexadecane were vortexed at
169 maximal speed (2400 rpm) for times ranging from 5 s to 600 s (and up to 1800 s when absorbance
170 continued to decrease after 600 s agitation) and left to settle for 30 min to allow complete
171 separation of the two phases. To prevent any variability between operators and to allow the

implementation of long vortexing steps, i.e. of 15 min or over, a specific device was designed in the laboratory to maintain the tubes in position during the mixing step (Figure 3A).

The absorbance of the aqueous phase at 600 nm was measured before mixing (A_0) and at different vortexing times (A_t). [(A_t/A_0)*100] was plotted against the vortexing time (s). The hydrophilicity/hydrophobicity was evaluated using the Gibbs partitioning energy (ΔG_{par}), calculated from A_{eq}, taking the asymptotic or the lowest absorbance value. Indeed, ΔG_{par} is obtained from the equilibrium constant K (ΔG_{par} = LnK), which expresses the maximal partitioning of bacteria between the aqueous and hexadecane phases. This was calculated from the equation $K = [6(A_0 - A_{eq}) / A_{eq})]$. The factor 6 in the equation was used to correct for the different volumes of the aqueous and hexadecane phases [21]. When hydrophilic particles were analyzed, very little removal from the aqueous phase, if any at all, was observed, resulting in low ΔG_{par} values. Conversely, when slightly hydrophilic, or hydrophobic particles are analyzed, low A_{eq} values are obtained, resulting in high ΔG_{par} values. In this study, a particle was considered to be hydrophobic for ΔG_{par} values >4.0, moderately hydrophilic for ΔG_{par} values ranging from 3.0 to 4.0 and highly hydrophilic for ΔG_{par} values <3.0.

2.4. Analysis of data and statistical analysis

Data were analysed by general linear model procedures using SAS V8.0 software (SAS Institute, Gary, NC, USA). Variance analysis was performed to determine the role of particle diameter on the hydrophobic measurement. These analyses were followed by multiple comparison procedures using Tukey's test (Alpha level = 0.05).

3. Results

Prior to experiments, preliminary works were carried out to define the experimental conditions under which relevant and reproducible results may be achieved (water contact angle). The influence

of the following parameters was first evaluated: (i) the filter pore size, (ii) the role of the addition of glycerol to the agar during the equilibration step, (iii) the efficiency of this equilibration step, (iv) the use of double-sided adhesive tape to maintain the filter on the glass slide, and lastly (v) the temperature and duration of the drying step. Below, we describe the evaluation of each of these parameters.

(i) Although the use of 0.45 µm pore size seems to be the subject of consensus [22,23], we investigated the appropriateness of using such filters in this study. Indeed, *B. pumilus* spores and microspheres characterized by similar or even smaller sizes were analysed in this study. As suspected, these small features were able to pass through the 0.45 µm-filter. Therefore, further experiments were carried out with filters with a 0.22 µm pore size.

(ii) Views reported in the literature concerning the setting up of an equilibration step are more disparate. In our laboratory, preliminary works have shown that the bacterial lawn sometimes became detached from the filter during drying (data not shown). We therefore assessed the benefits of implementing an equilibration step. This step was performed by placing the filters covered with the bacteria or microspheres lawns on agar for a given time [22,24], whether supplemented with glycerol or not. The addition of glycerol to agarose 2% induced changes in the contact angle values on YG microsphere layers (1 µm diameter): the water contact angle decreased from around 100° to 5-10° when glycerol was added, as previously reported in the literature [25].

(iii) We compared the contact angle values obtained with or without the equilibration step (no addition of glycerol). Contact angles of around 100° were obtained with or without the equilibration step, suggesting that this step was needless. Conversely, a filter-drying step is of course required to remove excess moisture.

(iv) In order to avoid filters curling during drying, filters were fixed to glass coupons using double-sided adhesive tape. In order to check whether the solvents contained in the adhesive tape were able to cross the filter and further contaminate the microsphere/spore lawn, water

contact angles were measured directly on filter fixed on not on the glass coupons. A clear decrease in the values of the water contact angle was observed, which could be attributed to the presence of solvents in the double-sided adhesive tape. For further works, filters were held in place with small pieces of adhesive tape on the edge of the filter to prevent any solvent contamination.

(v) Lastly, the influence of temperature during the drying step was investigated. We observed that drying at 30°C or above may result in the tearing of the lawns or in the upward curling of the filter (Figure 2A, bottom), whereas flat lawns were obtained at 20°C (Figure 2A, top). Consequently, further experiments were carried out without any equilibration step and the 0.22 µm filters were dried at 20°C for over 1 h.

3.1. Assessment of microsphere hydrophobicity

Water contact angle measurements were performed on microsphere lawns (three independent experiments, five measurements per experiment). In the chosen experimental conditions, stable and reproducible water contact angles were obtained. For all diameters, both panels of microspheres exhibited very close contact angles, between 108.4° and 117.2° for the Polybeads and between 110.8° and 115.6° for the YG microspheres, suggesting that all the microspheres were clearly hydrophobic (Table 1). The variance analysis indicated that the water contact angle was not significantly affected by the YG microspheres diameters (p-value = 0.0934). Conversely, very small yet statistically significant differences were observed between Polybeads of different diameters (p-value < 0.0001), yet the Tukey's grouping did not correlate with diameter, indicating that the observed differences were not linked to the microsphere diameter.

Microsphere diameter (µm)	**YG microspheres**		**Polybeads**	
	θ_{water}	**Tukey's**	**θ_{water}**	**Tukey's**

	(average values)	grouping	(average values)	grouping
0.2	115.6 *(5.3)*	na	114.8 *(2.5)*	A
0.5	110.8 *(3.7)*	na	112.9 *(2.0)*	ABC
0.75	109.3 *(1.0)*	na	108.4 *(1.4)*	C
1.0	111.0 *(5.4)*	na	114.2 *(3.3)*	AB
2.0	107.9 *(1.2)*	na	108.8 *(2.5)*	C
3.0	112.8 *(3.5)*	na	117.2 *(1.4)*	A
4.5	-	-	109.9 *(2.1)*	BC

[a]Tukey's grouping (groups in the same column with common letters are not significantly different)
-: not determined; na: not applicable (p-value>0.005)
In brackets: standard deviation (n= 15)

Table 1. Water contact angles on microsphere lawns (YG microspheres and Polybeads). Average values and standard deviations calculated from 15 measurements. According to the variance analysis, the water contact angles measured on the lawns made of YG microspheres of different diameters were not significantly different. Tukey's grouping was therefore only carried out on the Polybeads.

Microsphere hydrophobicity was also assessed through the MATH method for shaking times up to 30 min. As shown in Figure 3B and in Figure 4A, wide differences were observed in the results of the different Polybeads microspheres devoid of staining. The Polybeads' behaviour was deeply affected by their diameter, although a decrease in the aqueous suspension absorbance was clearly observed, even with the smallest microspheres. For both Polybeads and YG particles, the $(A_t/A_0)*100$ ratios converge towards an asymptote around zero for the largest microspheres, which means that almost all microspheres were adsorbed at the interface between water and hexadecane. Conversely, for the smallest microspheres (0.2 µm and 0.5 µm) no asymptote was reached after 30 min of shaking. Further experiments were performed with longer shaking times (up to 2 h), but again, no asymptote was reached. For example, the absorbance of the aqueous suspension of the 0.2 µm microspheres continued to decrease between 90 min and 120 min of shaking, to reach 70% of the initial absorbance after 2 h. In such cases, the equilibrium constant K was therefore calculated from the

minimum values obtained after 30 min shaking. Thus, LnK values first increased with the diameter (up to 1.0 µm) and then remained more or less steady at values between 4.8 and 6.0 (Table 2).

Microsphere diameter	YG microspheres		Polybeads	
	LnK (average values)	Tukey's grouping	LnK (average values)	Tukey's grouping
0.2 µm	-2.60 *(2.6)*	C	1.01 *(0.4)*	C
0.5 µm	-0.73 *(0.4)*	C	1.91 *(1.1)*	BC
0.75 µm	6.13 *(0.4)*	AB	4.08 *(0.8)*	AB
1.0 µm	5.11 *(1.5)*	B	4.80 *(1.3)*	A
2.0 µm	4.59 *(0.6)*	AB	4.74 *(2.4)*	A
3.0 µm	8.22 *(0.0)*	A	6.07 *(0.5)*	A
4.5 µm	-	-	4.91 *(2.5)*	A

[a]Tukey's grouping (groups in the same column with common letters are not significantly different)
-: not determined
In brackets: standard deviation (n= 3 to 8)

Table 2. Affinity of the microspheres to hexadecane (YG microspheres and Polybeads). Average values and standard deviations calculated from 3 to 8 measurements. According to the variance analysis, the LnK values of the YG microspheres of different diameters were significantly different, as were those of the Polybeads of different diameters. Tukey's grouping was therefore carried out on both microspheres.

These observations on Polybeads were confirmed by variance analysis (p-value = 0.0003) and 66% of the variability in LnK (or in other words the microsphere concentration of the aqueous suspension after vortexing) was explained by the microsphere diameter (r^2 = 0.6576). The Tukey's grouping shown in Table 2 is consistent with the above information. Those microspheres with a diameter greater than or equal to 1.0 µm were considered as hydrophobic, while the 0.2 µm and 0.5 µm-diameter microspheres were considered as highly hydrophilic. Lastly, the 0.75 µm-diameter microspheres had intermediate properties (group AB according to Tukey, which were therefore not significantly different from Group A [1.0 µm-microspheres] or from Group BC [0.50 µm-microspheres]).

292 Further experiments were carried out with fluorescent microspheres (YG), which were supposed
293 to exhibit different surface properties, based on previous results obtained with the 0.5 µm-diameter
294 YG microspheres (data not shown). As reported above for Polybeads, the diameter seems to strongly
295 influence the microsphere's behaviour towards hexadecane. Results are shown in Figure 4B. When
296 the smallest YG microspheres (0.2 µm and 0.5 µm) were tested, A_t/A_0 was practically constant,
297 resulting in very low LnK values, thus suggesting a strongly hydrophilic character. Conversely, the LnK
298 values indicated a strong hydrophobic character for all other YG microspheres. Furthermore, for
299 diameters greater than or equal to 1.0 µm, the larger the microsphere, the quicker the absorbance
300 decrease in the aqueous suspension, while results obtained with the 0.75 µm-YG microspheres were
301 less accurate. Finally, variance analysis confirmed that the microsphere diameter strongly affects LnK
302 (p-value < 0.0001), with this parameter accounting for 95% of the whole LnK variability. According to
303 the Tukey's grouping (Table 2), the smallest microspheres (0.2 µm and 0.5 µm diameter) are
304 significantly more hydrophilic than the other microspheres.
305
306 *3.2. Assessment of spore hydrophobicity*
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308 We used the same two methods to characterize the hydrophobic properties of *Bacillus* spores.
309 Unlike with microsphere lawns, water contact angles differed according to bacterial strain. Six of the
310 eight spores were highly hydrophobic, with water contact angles exceeding 100° (Table 3). Only Bc
311 D6 and Bs 7135 were respectively considered as moderately hydrophilic and hydrophilic. The
312 statistical analysis confirmed that the contact angle of the spores depends on the *Bacillus* strain ($p <$
313 0.001). The Tukey's grouping further distinguished between more or less hydrophobic spores. Among
314 the six hydrophobic spores, Bc 98/4 and Ba 9131 were thus considered significantly more
315 hydrophobic than Bp 98/6 and Bt 7138. A similar trend seems to emerge from the data obtained by
316 MATH and shown in Table 3. For example, Bc 98/4 was the most hydrophobic and Bs 7135 the most
317 hydrophilic strain for both methods. However, it may be noted that there are key differences

between the results obtained with the two methods. Indeed, Bc 14579 spores were amongst the most hydrophobic spores according to the contact angle measurement, but not according to the MATH method.

Spore strains	θ_{water}		LnK		Spore sizes	
	Average values	Tukey's grouping	Average values	Tukey's grouping	Length*	Width
Bc 98/4	111.3 *(3.2)*	A	7.28 *(1.0)*	A	1.57	0.83
Ba 9131	111.0 *(2.3)*	A	5.00 *(0.7)*	B	1.32	0.78
Bc 14579	107.8 *(1.3)*	AB	3.45 *(0.3)*	D	1.22	0.63
Bt 407	107.7 *(1.0)*	AB	6.32 *(1.3)*	A	1.27	0.68
Bp 98/6	103.9 *(5.0)*	B	4.75 *(0.9)*	BC	0.96	0.51
Bt 7138	102.8 *(2.1)*	B	2.66 *(0.1)*	DE	1.20	0.70
Bc D6	71.1 *(1.4)*	C	3.71 *(0.2)*	CD	1.29	0.68
Bs 7135	44.8 *(3.0)*	D	2.08 *(0.6)*	E	1.15	0.61

**not taking account of exosporia*
[a]Tukey's grouping (groups in the same column with common letters are not significantly different)
In brackets: standard deviation (n= 4 to 8)

Table 3. Water contact angles on *Bacillus* spore's lawns and affinity of the spores to hexadecane (LnK). Average values and standard deviations calculated from 4 to 8 measurements. According to the variance analysis, significant differences were obtained between the hydrophobic characters of spores. Tukey's grouping was therefore carried out on both parameters.

4. Discussion

Unlike colloidal particles, bacteria have complex and heterogeneous surfaces with a variety of structural features, resulting in complex microbe-surface interactions. Even bacterial spores, although dormant, have more or less complex surfaces, with the presence both of polymeric layers including polysaccharides [9], and surface features such as appendages [10]. We thus used calibrated latex microspheres with diameters between 0.2 µm and 4.5 µm, to evaluate whether their

hydrophobic character could be affected by their size. Indeed, very similar water contact angles were obtained whatever the microsphere size. Moreover, the contact angles were very high (about 110°), indicating a pronounced hydrophobic character.

One of the major advantages of MATH analysis is that this method is very easy to implement. Conversely, the results are influenced by the mixing conditions and consequently, it is difficult to compare data from different laboratories. Despite this potential problem, this method is widely used in microbiology, since it does not require any expensive material. Given the high contact angles, one would expect that during the vortexing step of the MATH method, the microspheres would quickly migrate to the interface, resulting in a quick absorbance decrease of the aqueous phase. In reality, a sharp decrease in the absorbance of the microsphere suspension was clearly observed when the microsphere with diameter > 0.75 µm were tested. Conversely, for diameters lower than or equal to 0.75 µm, the absorbance decreased only slowly or even not at all (YG microspheres of 0.2 and 0.5 µm diameters). Considering the identical chemical surface properties of all microspheres, the different absorbance obtained therefore seems to suggest that the MATH method may not be suitable for the analysis of particles smaller than one micron. At the current stage of study, we cannot provide a physical explanation for this observation, but can only report the distinct results when particle size is smaller than one micron. This will be the subject of further experimental and theoretical investigations of the absorbance mechanism of particles of smaller than 1 µm using the MATH method. Elsewhere, even for the microspheres with diameter > 0.75 µm, the lowest absorbance values (Aeq) were reached for shaking times over 5 min and the smallest the microsphere, the longer the time to reach Aeq. Therefore, the short vortexing times reported in the literature (down to 30 s [26] or even 10 s [13]) would probably result in the underestimation of the hydrophobic character of the tested particles.

The influence of the particle size having been demonstrated on simple models, we then investigated whether a similar phenomenon could be detected on bacteria, making it difficult to estimate their hydrophobicity. In this study, *Bacillus* spores (wet densities around 1.20 $g.cm^3$ [27])

were preferred to vegetative cells, because spore surface is relatively stable over time, contrarily to vegetative cells, whose surface structures are neither spatially nor temporally constant and vary with changes in environmental conditions, e.g. in response to adhesion to inert surfaces [28]. The measurement of the water contact angle as well as the calculation of LnK indicated that the bacterial spores were hydrophilic to hydrophobic, in accordance with previous results on the contact angles of *Bacillus* spores [24] and mainly on the adhesion of spores to hexadecane [29–31]. However, discrepancies were observed between the two methods, mainly concerning Bc14579 and Bt7138, which were assumed to be hydrophilic by MATH and hydrophobic by goniometry. As clearly shown in Figure 5, Bc14579 and Bt7138 spores were medium-sized, and these discrepancies could not be attributed to the influence of the spore size. Such discrepancies have been already pointed out [32], and the authors suspected the role of appendages and/or superficial macromolecules. It can also be assumed that they may be due to the presence of the loose balloon-like envelope called exosporium surrounding spores belonging to the *B. cereus* group (but very small on *B. anthracis* spores) and absent from spores belonging to other species such as *B. subtilis* or *B. pumilus* [10]. Information obtained with the MATH method should thus be considered with a degree of caution and must be validated by the measurement of the water contact angles.

5. Conclusion

In this study, we first clearly demonstrate that the size of latex particles strongly affects the results of a MATH evaluation hydrophobicity. Indeed, despite their pronounced hydrophobic character according to contact angle measurements, the smallest microspheres remained in the aqueous phase after 30 min shaking and were thus regarded as hydrophilic by the MATH method. The experimental results seem to suggest that within the tested particles of densities close to their environing fluid, the MATH method may not be suitable for the analysis of particles or bacteria under 1 µm in diameter. In this regard, further studies are needed to investigate the mechanism behind the

particle absorption in the MATH method. Such a study should, in particular, investigate the conditions required for a particle (relative fluid-particle velocity, wetting property, size, required contact time with the interface for an interface absorption, etc.) to be absorbed by a hexadecane droplet.

A similar approach applied to a panel of *Bacillus* spores, considered as hydrophilic to hydrophobic according to the water contact angles, was inconclusive as to the influence of the spore size, perhaps due to the insufficient differences in their sizes (lengths ranging from 0.96 to 1.57 µm). However, some discrepancies observed with the water contact angles indicate that the information obtained from the MATH method should be considered with a degree of caution, perhaps due to the complexity of the spore surface. Furthermore, when the hydrophobicity of microorganisms of different sizes (e.g. small bacteria belonging to the *Bordetella* or *Chlamydia* genus vs large bacteria such as *Sarcina* or yeasts) has to be assessed, we would recommend the use of the contact angle measurement. Lastly, since it has been demonstrated that the shape of particles, e.g. isotropic [spheres] vs anisotropic [ellipsoids to tubes] particles, dramatically affects their behaviour at the air-liquid interface [33,34], it could be interesting to investigate if the bacterial shape (cocci/bacilli/filaments) could affect their behaviour during the shaking step of the MATH method.

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Figure captions

Figure 1. Surface composition of YG Fluoresbrite® microspheres by Fourier-transform infrared spectroscopy (FTIR). Comparison of spectra from YG Fluoresbrite® and Polybeads® microspheres of various diameters (0.2, 1.0, 3.0 µm) with the spectrum of ATR polystyrene from the data base (black).

Figure 2. Microsphere and spore lawns (Fig. 2A) dried at optimal temperature (Top: YG microspheres dried at 20°C, resulting in a regular lawn; Bottom*: B. pumilus* spores dried at 30°C, resulting in the presence of irregularities on the lawn surface). Fig. 2B: examples of water contact angles.

Figure 3. Specific device (Fig. 3A) designed to maintain the tubes in position during the mixing step of the MATH protocol. Tubes contain 3 ml of the aqueous suspension and 0.5 ml of hexadecane. Turbidity of the aqueous suspension observed when the Polybeads were tested (Fig. 3B), after complete separation of the two phases.

Figure 4. Examples of data obtained from the MATH method on Polybeads (A) and YG microspheres (B). The hydrophobicity is estimated from the reduction of the turbidity of the aqueous suspension of microspheres as a function of time.

Figure 5. Spore hydrophobicity estimated by MATH (LnK, □) and goniometry (water contact angle, ■) as a function of the spore length (A) and width (B). Dashed line: threshold value between hydrophobic and hydrophilic spores

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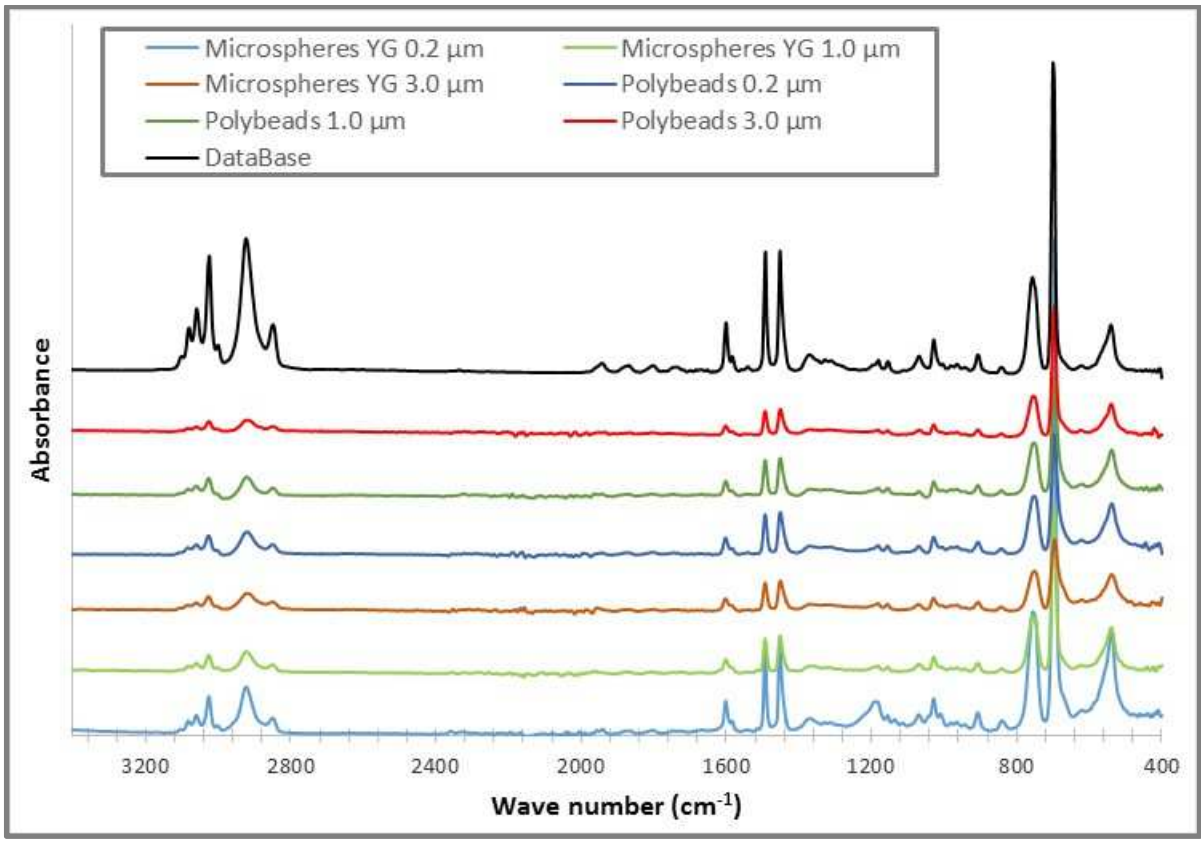

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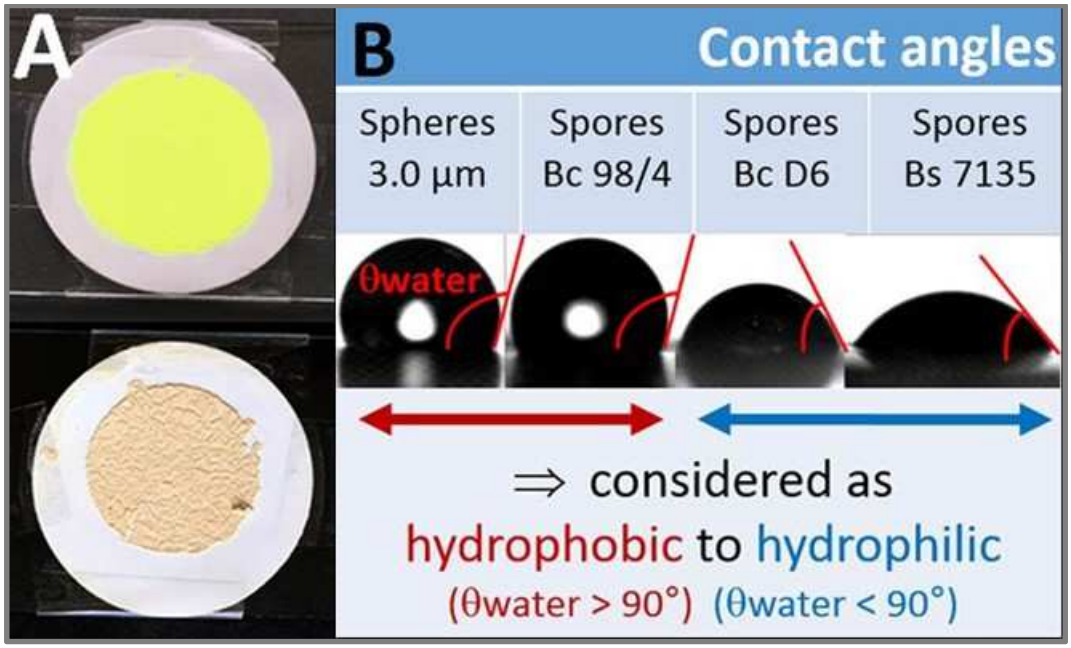

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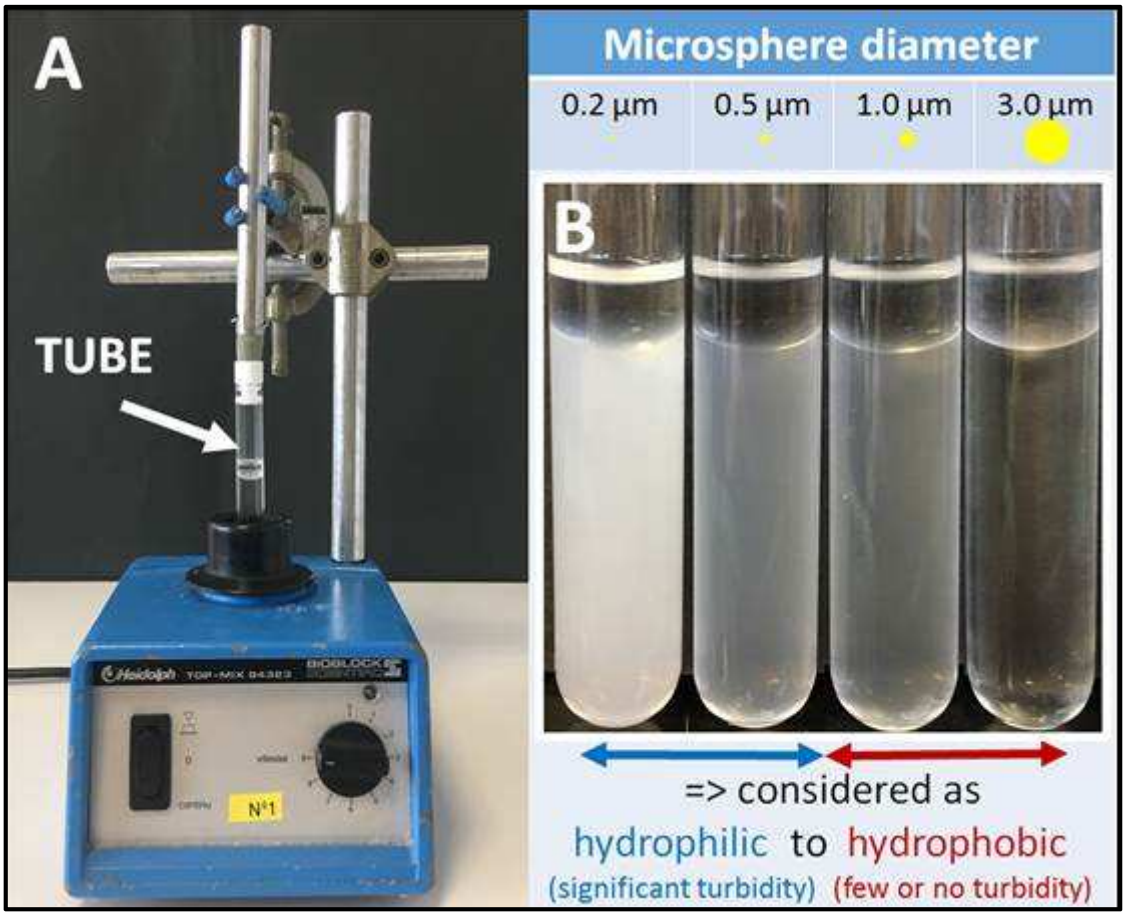

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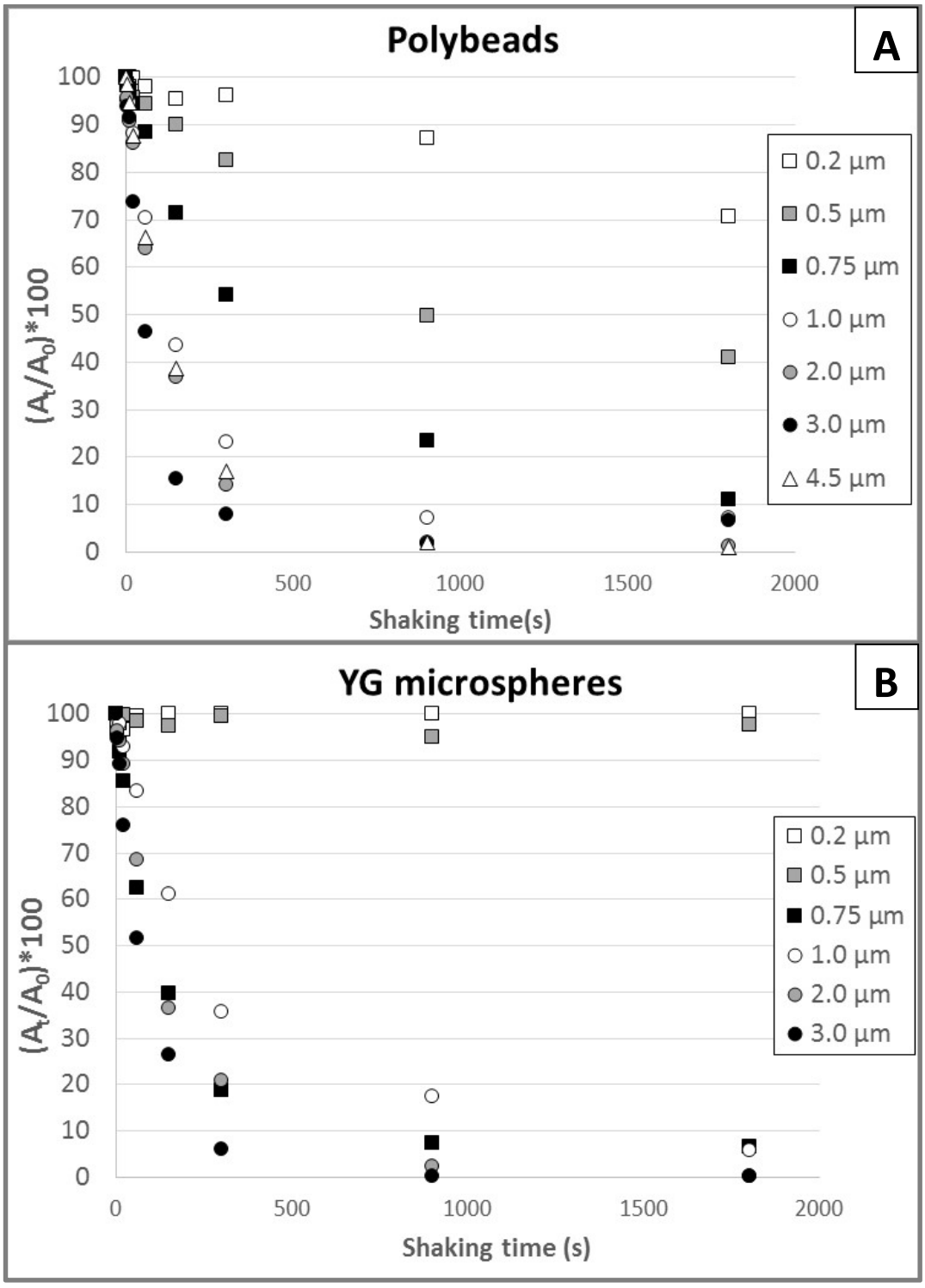

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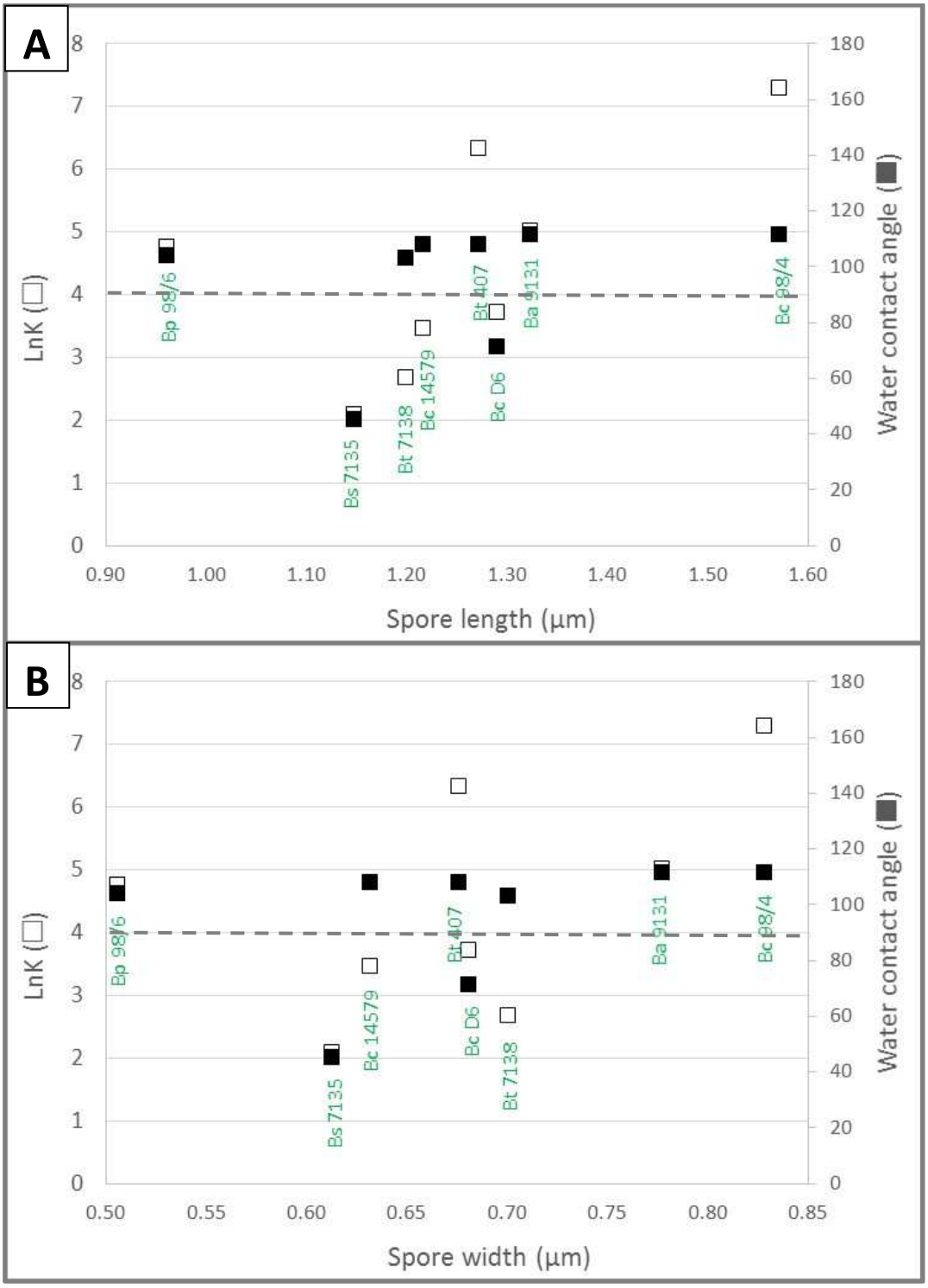
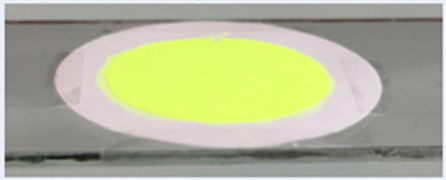


Assessment of microsphere hydrophobic/hydrophilic character

Microsphere diameter

0.2 µm	0.5 µm	1.0 µm	3.0 µm

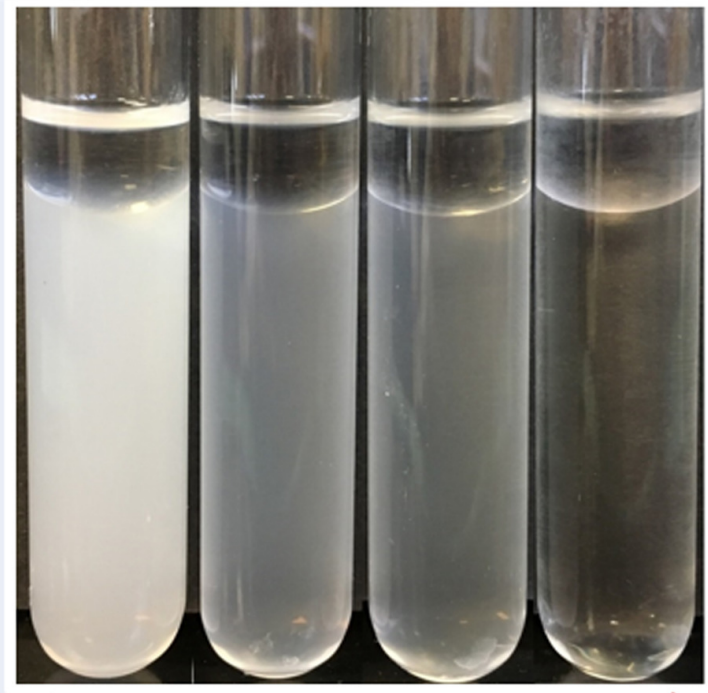
According to the water contact angle on a microsphere lawn



=> considered as hydrophobic

(θwater ≈ 110°)

According to the MATH method (residual trouble of the aqueous suspension of microspheres after 5 min-mixing)



=> considered as

hydrophilic / hydrophobic

(significant turbidity) / (few or no turbidity)