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1 **For submission to Journal of Microbiological Methods**

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3 **Rapid and simple As(III) quantification using a turbidimetric**
4 **test for the monitoring of microbial arsenic bio-**
5 **transformation**

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11

12 **Abstract**

13 A turbidimetric test for rapid quantification of As(III) (detection limit of 3 mg/L, quantification range of
14 10-100 mg/L) in liquid growth medium was developed for assessing and monitoring microbial As(III)-
15 oxidizing and As(V)-reducing activities. This test is based on As(III) chelation with pyrrolidine
16 dithiocarbamate followed by spectrometric measurement of absorbance, and was validated by
17 comparison with AAS quantification of As after As(III)/As(V) separation.

18 **Key words:** arsenite quantification, pyrrolidine dithiocarbamate (PDTTC), turbidimetric method,
19 laboratory scale analysis, arsenic bio-transformation.

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21 Arsenite (As(III)) is a major pollutant in many countries and is responsible of major sanitary problems
22 due to the use of As-rich groundwater or surface water as drinking water. In view of the bioremediation
23 of As(III)-polluted sites based on microbial As(III) oxidation into arsenate (As(V)) that precipitates more
24 easily, many researches are directed toward the isolation and characterization of microbial strains and
25 consortia able to oxidize As(III) (Santini et al., 2000 ; Battaglia-Brunet et al., 2002; Ike et al., 2008 ;
26 Osborne et al., 2010 ; Paul et al., 2018). These works are generally screening for microorganisms highly
27 tolerant to As and are usually conducted at high As(III) concentrations (until 100 mg/L and even more)
28 and need analytical tools that allow total As quantification and also As(III) and As(V) species
29 quantification at high concentrations. Standard analytical methods commonly used for arsenic (As)
30 quantification (atomic absorption spectroscopy, mass spectroscopy) are sensitive with low detection
31 limits and allow redox state determination. However, their main drawbacks are the equipment cost,
32 the need of specific qualification of the experimenters, and the need to transport and store samples
33 from the site to the laboratory. Several colorimetric tests have been developed to overcome these
34 drawbacks (Dhar et al., 2004 ; Baghel et al., 2007 ; Hu et al., 2012 ; Sidhu et al., 2014; Sirawatcharin et
35 al., 2014; Rathore, 2017 ; Kearns and Edson, 2018 ; Lace et al., 2019). They are cheap methods that
36 allow routine and high frequency monitoring of polluted sites (that can be used as alert systems), and
37 screening of samples that need to be further analyzed by standard analytical methods. However, as
38 most of these colorimetric methods have been developed for on-site measurements of As in drinking
39 waters, they usually display low detection limit and quantification ranges in the $\mu\text{g/L}$ order, and for
40 many of them, redox state determination (As(III) vs. As(V)) is not possible. With the exception of few
41 of them (Hu et al., 2012), most of these methods are not adapted to the characterization and
42 monitoring of microorganisms in a context of site bioremediation. There is thus a need for rapid and
43 simple laboratory tests that allow the quantification of As species, such as As(III), at high
44 concentrations (until several dozens of mg/L).

45 The objective of this work was to develop a simple and rapid method to quantify As(III) (but not As(V))
46 in the 0-100 mg/L range in microbial culture media in the frame of growth, selection and

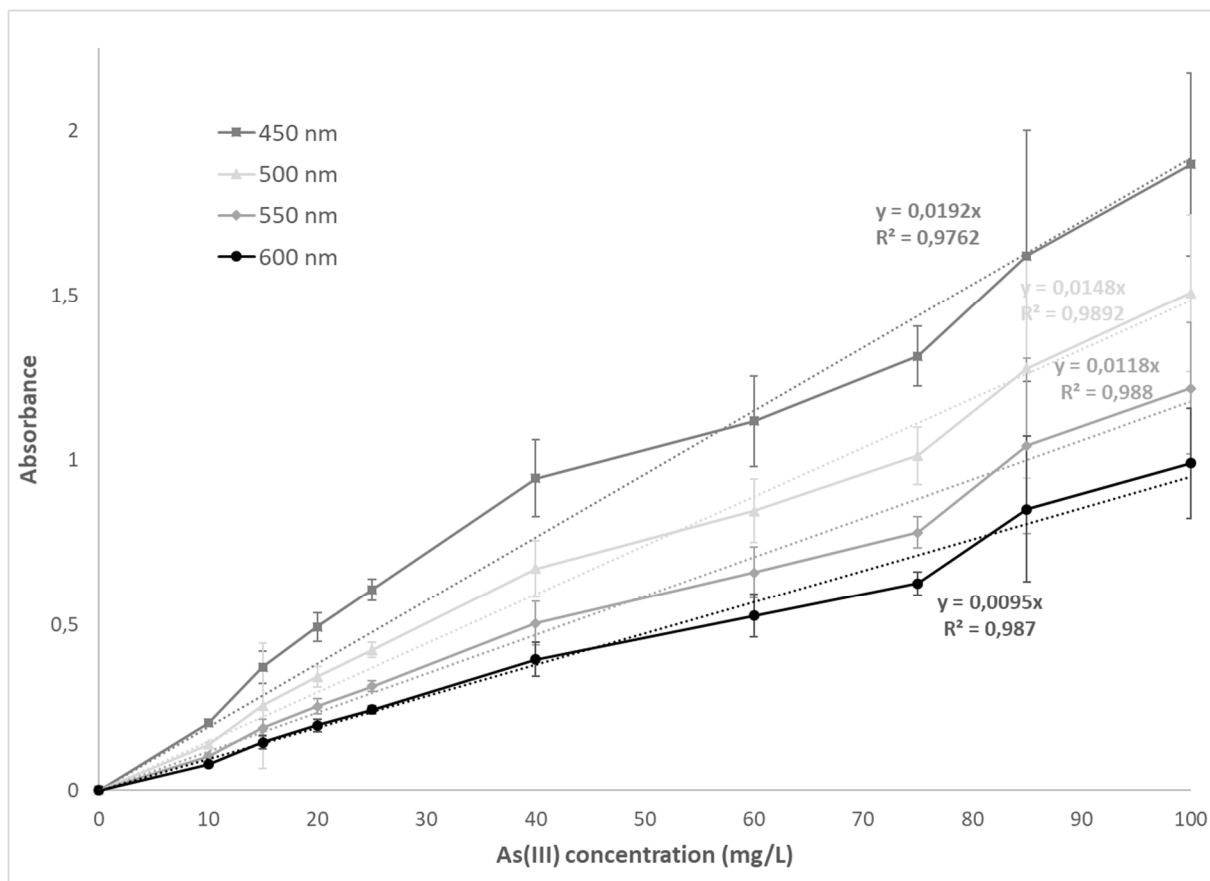
47 characterization of strains and consortia that express As(III)-oxidizing activity. In this context, the
48 possibility to use the metals/metalloids chelation properties of pyrrolidine dithiocarbamate (PDTC)
49 ($C_5H_9NS_2$) was evaluated. This chemical compound is known to form a non-soluble complex with As(III)
50 and not with As(V) at pH 5, thus allowing to determine the redox state of As (Charlot, 1974;
51 Subramanian and Meranger, 1981).

52 The procedure for the proposed PDTC test is simple and rapid (per sample: 5 minutes from sample
53 preparation to absorbance measurement with a UV-visible spectrophotometer). In a glass tube, 0.5
54 mL of filtered growth medium (at 0.22 μ m) are mixed with 0.5 mL of acetate buffer (0.1 M Na-acetate,
55 pH 5 (pH is adjusted using acetic acid)). Then, 0.1 mL of a PDTC stock solution (5 g of PDTC (Sigma)
56 dissolved in 1 L of demineralized water) are added and immediately mixed by a rapid hand shaking (by
57 inverting the tubes 2-3 times). In the presence of As(III), white small precipitates immediately appear
58 (whereas there are no precipitates with As(V)). Absorbance (at 450, 500, 550 and 600 nm) is then
59 measured using a spectrophotometer (in this study: Cary 100 UV-Vis spectrophotometer, Agilent
60 Technologies, using 1 cm light path cuvettes). Absorbance measurements have to be done immediately
61 after shaking, as the precipitates tend to aggregate after 1 minute. It is thus recommend to analyze
62 one sample at a time. The acetate buffer and PDTC stock solutions can be stored at room temperature
63 for several months.

64 The first step of this work aimed to validate the possibility to quantify the turbidity due to As(III)-PDTC
65 interaction using spectrophotometry. For this, the PDTC test was applied on As(III) solutions at various
66 concentrations ranging from 0 to 100 mg/L and prepared in distilled water. Results showed a linear
67 relation (R^2 about 0.98) between absorbance and As(III) concentration for the four tested wavelengths,
68 and for As(III) concentrations ranging from 0 to 100 mg/L (Fig. 1). The calibration curves varied
69 according to the wavelength so that the sensitivity was the highest at 450 nm and the weakest at 600
70 nm (Fig. 1, Table 1). The potential influence of microbial growth medium composition (as the presence
71 of salts) on the sensitivity was then evaluated. For this, As(III) solutions prepared in CASO1 medium (a

72 defined growth medium used for the growth of As(III)-oxidizing bacteria, Battaglia-Brunet et al., 2002)
73 or 2A-MOPS medium (a defined growth medium adapted to marine As(III)-oxidizing bacteria, Lescure
74 et al., 2013) were tested. Results demonstrated the influence of growth medium composition as
75 sensitivity and quantification range varied according to the medium (Table 1). Sensitivity was indeed
76 slightly higher for the CAsO1 medium in comparison to results obtained with distilled water and 2A-
77 MOPS medium. This is potentially due to the presence, in the CAsO1 medium only, of Mg^{2+} and Ca^{2+}
78 that probably favored the flocculation of precipitates (Sworska et al., 2000). Same sensitivity and
79 detection range were obtained in the CAsO1 medium prepared with or without yeast extract (data not
80 shown), showing no impact of yeast extract on the quantification of As(III). Concerning quantification
81 range, for the CAsO1 medium, the linear relation between As(III) concentration and absorbance was
82 lost for concentrations higher than 40 mg/L, at which a plateau was observed (data not shown)
83 because of high absorbance values incompatible with the Beer-Lambert equation. To overcome this
84 bias, if observed, a dilution of the sample, using growth medium as the dilution solution, is needed
85 before analysis with the PDTC test. For the 2A-MOPS medium, As(III) quantification was possible until
86 100 mg/L range. More precisely, the detection limit was about 3 mg/L in water and 2A-MOPS medium,
87 and about 2 mg/l in CAsO1 medium. However, linearity was optimal for concentrations higher than 10
88 mg/L. Taken all together, these results demonstrated the relevance of the PDTC test for quantifying
89 As(III) in culture media containing up to 100 mg/L of As(III) (according to the growth media), and even
90 more by diluting the sample. For accurate sensitivity of the method, the calibration curves have to be
91 done in the growth medium used for the studied microbial strain or consortium. Moreover, to have
92 the best As(III) concentration determination, it is suggested to measure the absorbance at the 4
93 wavelengths to calculate an average As(III) concentration.

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Fig 1: Calibration curves obtained for the quantification of As(III) in distilled water by the PDTc test. Absorbance measurements (n=3) were registered at four different wavelengths (450 nm, 500 nm, 550 nm and 600 nm). Dotted lines represent trend lines that correspond to calibration curves. Bars represent standard deviation.

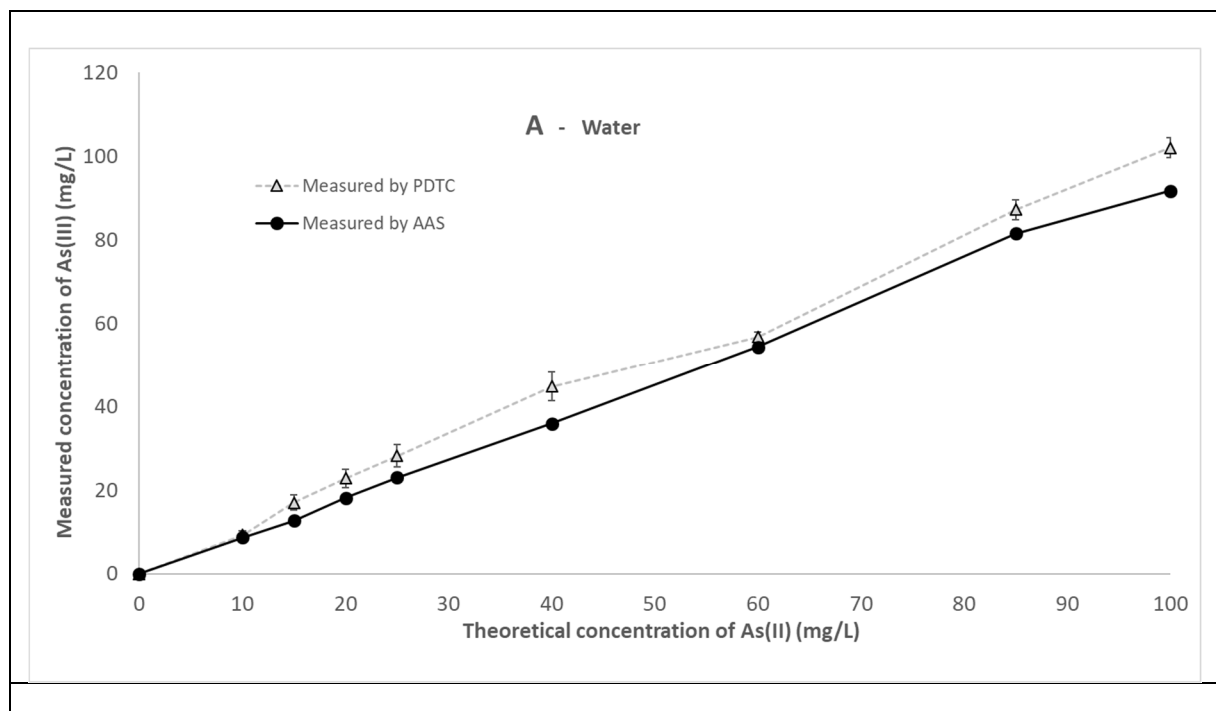
Wavelength (nm)	Sensitivity for As(III) (Abs / (mg/L))				Quantification range (mg/L)
	450 nm	500 nm	550 nm	600 nm	
In Water	0.0192	0.0148	0.0118	0.0095	10 - 100
In CAsO1 medium	0.0323	0.0232	0.0174	0.0136	10 - 40
In 2A-MOPS medium	0.0182	0.0141	0.0112	0.0092	10 - 100

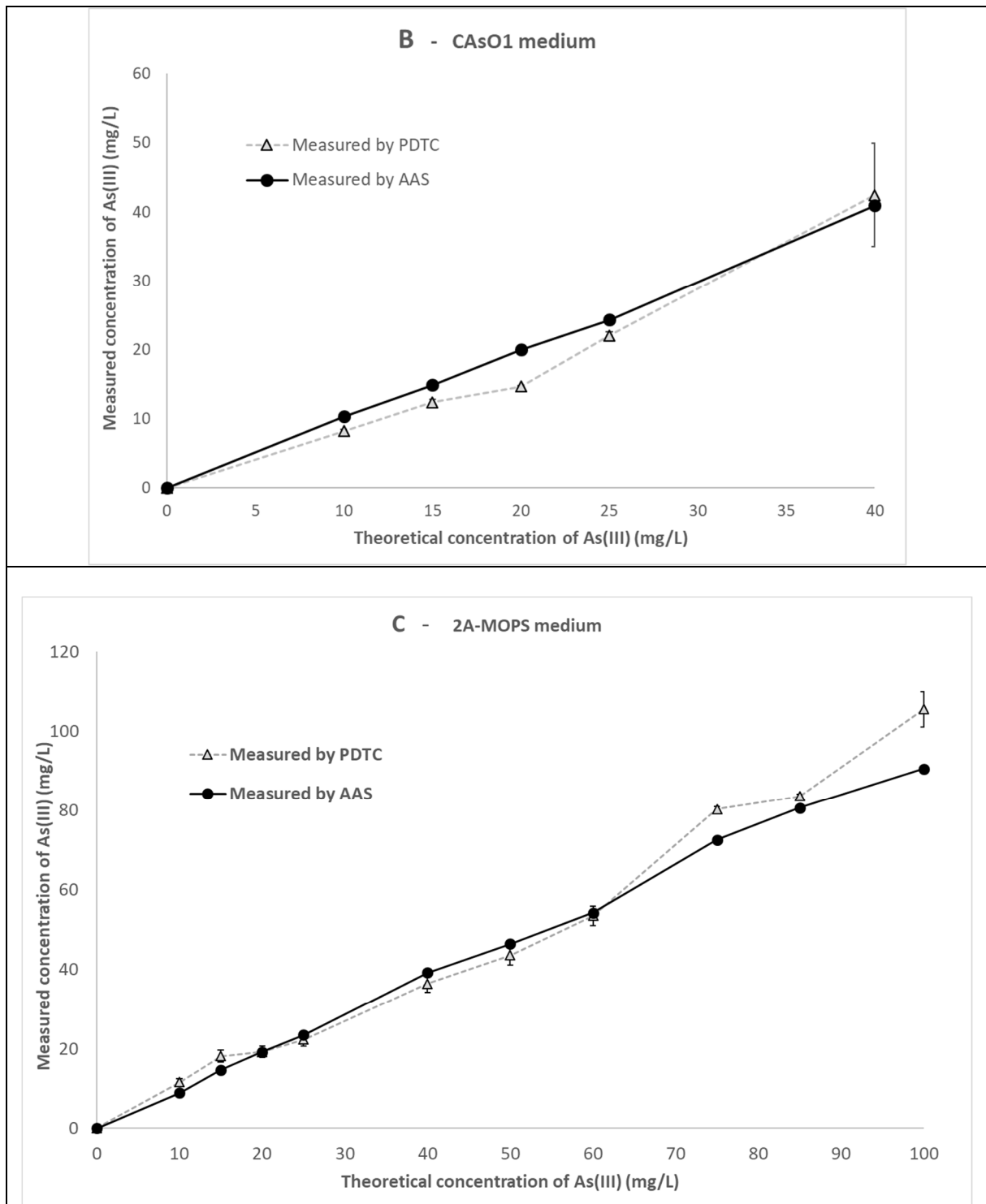
104 Table 1: Sensitivity and quantification range of the PDTc test for As(III) according to the medium
105 (CAsO1 medium, 2A-MOPS medium and distilled water) and to the wavelength used for absorbance
106 measurement. Sensitivity values were calculated from trend lines such as those presented for water in
107 Fig 1.

108

109 The PDTC test was then validated by comparison with a standard analytical method for As
110 quantification and routinely applied for the monitoring of As(III)-oxidizing or As(V)-reducing activity
111 tests (Lescure et al., 2016 ; Thouin et al., 2016). For this, As(III) was selectively extracted in Methyl
112 IsoButyl Ketone (MIBK) with PDTC, using a protocol adapted from Charlot (1974) and detailed in
113 Battaglia-Brunet et al. (2002). Total As and As(V) recovered in the aqueous phase were analyzed by
114 Atomic Absorption Spectrophotometry (AAS; Varian AA22FS). The detection limit of this method was
115 1 mg/L. Standard As(III) solutions from 0 to 100 mg/L were prepared in distilled water, CASO1 medium
116 and 2A-MOPS medium. As(V) and total As were quantified by AAS and As(III) was quantified by the
117 PDTC test. For quantification using the PDTC test, calibration curves obtained in Figure 1 and Table 1
118 were used for the calculation of As(III) concentrations from absorbance values. For the two growth
119 media and water, results from AAS and PDTC test showed similar results allowing the validation of the
120 PDCT test (Fig. 2).

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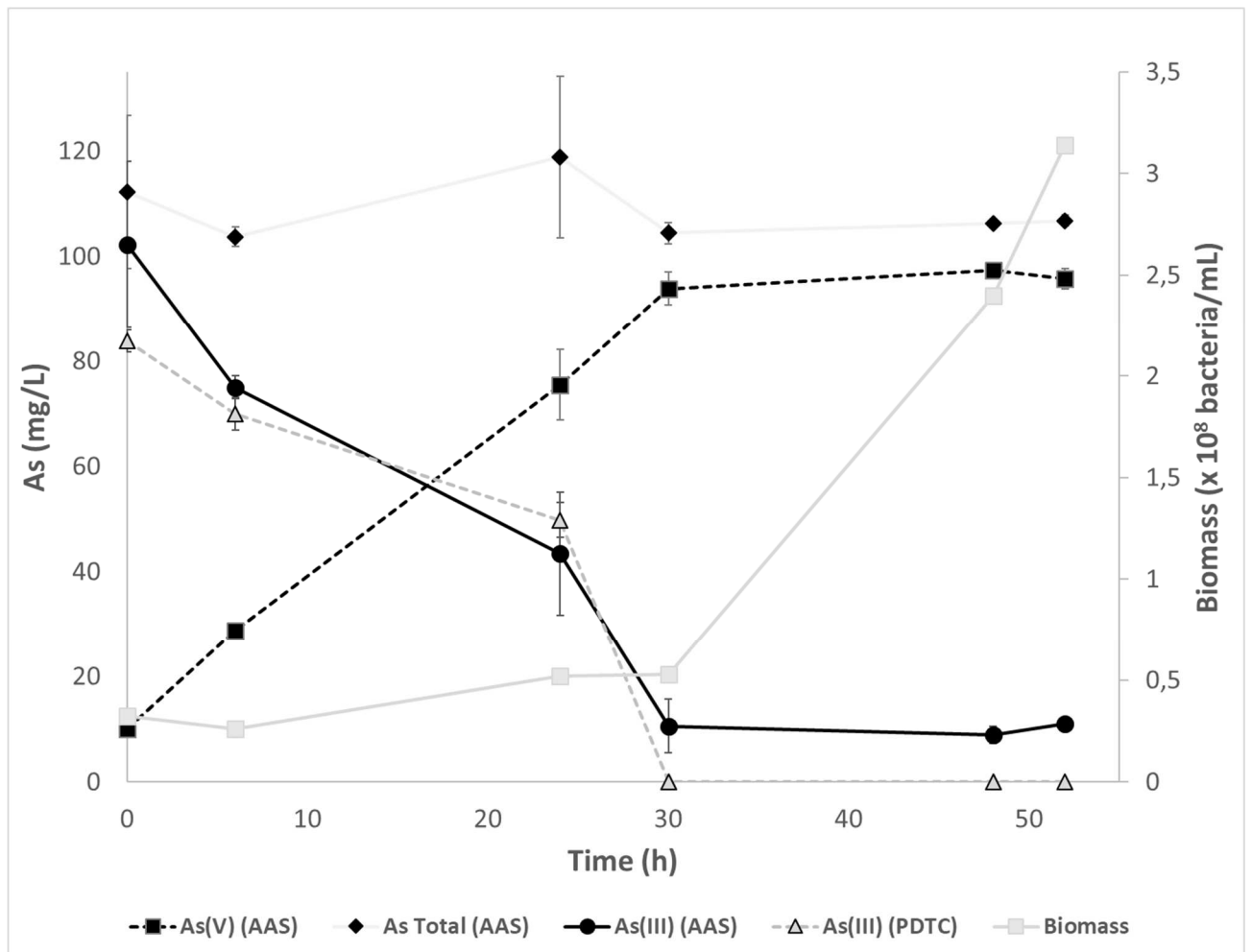


125 Fig 2 : Comparison of theoretical As(III) concentrations and measured As(III) concentrations by the
 126 PDTC test (squares, n=3) and AAS (circles, n=1). For the AAS approach, As(III) concentrations were
 127 obtained by deduction from total As and As(V) concentrations. Bars represent standard deviation.

129 Finally, the use of the PDTC test to measure and monitor As(III) oxidation by a bacterial strain was
130 evaluated. For this, the As(III)-oxidizing bacterium *Thiomonas delicata* subsp. *arsenivorans* (Battaglia-
131 Brunet et al., 2011) was grown in the CAsO1 medium at 25°C, in static conditions. Its growth was
132 monitored by microscopic cell counts (using a Thoma cell (Marienfeld, depth 0.1 mm), and by
133 examining 10 fields with a Zeiss Axio Imager Z1, magnification x400, AxioVision 4.6 software), and its
134 As(III)-oxidizing activity was monitored by measuring As(III) concentration in the culture medium using
135 the PDTC and AAS (for validation) approaches (Fig. 3). Abiotic tests (no inoculation) confirmed the
136 stability of As(III) at 25°C in the CAsO1 medium over time (data not shown). Results showed that the
137 As(III) oxidation kinetics was the same whatever the analytical method used for As(III) quantification,
138 thus confirming the possibility to use the PDTC test for monitoring the activity of microbial strains able
139 to oxidize As(III). Although not tested here, the developed test is also adapted to monitor the activity
140 of As(V)-reducing microorganisms, since As(III) is the end product during the biological reduction of
141 As(V).

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145 Fig 3 : Growth and As(III)-oxidizing activity of *Thiomonas delicata* subsp. *arsenivorans* grown in the
 146 CASO1 medium. As(III) was quantified using the PDTC test (n=3) (results are means of values obtained
 147 at 550 nm and 600 nm), and AAS was used to quantify total As and As(V), thus allowing by deduction
 148 to obtain As(III) concentration (n=3). Bacterial growth was monitored by bacteria counting using a
 149 Thoma cell. Bars represent standard deviation.

150

151 In conclusion, a rapid (5 minutes per sample), simple (one reagent and one step) and cost effective
 152 (reagent (PDTC) cost is about 60 € for the preparation of a 5 L solution) turbidimetric method was
 153 developed for the quantification of As(III) (but not As(V)) in the range of 0 to 100 mg/L (according to
 154 the growth medium) or even more due to the possibility to dilute samples. This kind of test is
 155 particularly adapted to the characterization of As(III)-oxidizing microbial strains or consortia, but also

156 to As(V)-reducing microorganisms, as it allows to easily quantify and monitor their As-linked activity.
157 This test, used here at the laboratory scale, could also be helpful at the (semi)-industrial scale for the
158 development and monitoring of bioprocesses (prototypes or industrial installations) for
159 bioremediation purposes such as treatment of As polluted water. Indeed, easy, cheap and rapid tests
160 are needed at all scales to monitor bioremediation process efficiency, alert if pics of As(III) pollution
161 are observed, and also select samples of interest that need further characterization using standard
162 protocols. A third application would be the easy and cheap determination of microbial As(III)-oxidizing
163 and As(V)-reducing activities in environmental samples, that proved to be interesting biogeochemical
164 indicators (Lescure et al., 2016; Thouin et al., 2016). At last, preliminary tests realized in our laboratory
165 also suggested that the developed PDTC turbidimetric test could be used to quantify Sb(III) (but with a
166 lower sensitivity than that obtained for As(III)), another metalloid oxidized by some bacteria (Wang et
167 al., 2015). PDTC was also demonstrated to interact with selenium (Subramanian and Meranger, 1981),
168 suggesting that the PDTC test could potentially be used for the characterization of the bioremediation
169 properties of microorganisms involved in the arsenic, antimony and/or selenium cycles.

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173

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