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1 The colorimetric assay of viability for algae (CAVA): a fast  
2 and accurate technique

3

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15

16

17 Abstract

18 Determining the fraction of viable cells in algal cultures is critical to improve the  
19 understanding and control of algal microbiology, ecology, and biotechnology. Whereas  
20 current techniques for algal viability determination can be rather cumbersome, this paper  
21 describes a new assay that enables the rapid quantification of algal viability using only  
22 spectrophotometric measurements. This technique, henceforth named CAVA test, relies on  
23 the selective adsorption of erythrosine by non-viable cells and was validated on the algal  
24 species *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. The results obtained by the  
25 CAVA test were in good agreement with the *in situ* measurement of oxygen production rates.  
26 In addition, the CAVA test was shown to quantify the viability of algal samples regardless of  
27 the cause of death (heating, UV-irradiation or H<sub>2</sub>O<sub>2</sub> exposure). The CAVA technique has  
28 therefore the potential to offer fast and universal approach to measure the viability of algal  
29 samples.

30

31 Keywords: Algae; *Chlorella vulgaris*; *Chlamydomonas reinhardtii*; Erythrosine; Mortality;  
32 Viability

33

34 1. Introduction

35 The ability to rapidly and accurately quantify the ratio of viable cells over total cells within  
36 microalgal cultures, henceforth defined as ‘viability’, is needed in various fields of algal  
37 research (e.g. ecology, Garvey et al. 2007). For example, it is critical to determine if a certain  
38 stressor (e.g. toxicant, Gilbert et al. 1992) causes cellular death rather than, or in addition to,  
39 activity inhibition to understand the mechanisms involved. The determination of viability is  
40 therefore critical to improve the design and control of algal biotechnology as algal cells found  
41 in full-scale cultivation systems are unlikely to be 100% viable due to, typically, viral-induced  
42 death (Garza and Suttle 1998), mechanical stress (Brindley Alías et al. 2004; Gudín and  
43 Chaumont 1991), UV-irradiation (Häder et al. 1997) and rapid temperature changes (Béchet  
44 et al. 2010).

45  
46 Various experimental techniques enabling the quantification of algal cells viability have been  
47 described in the literature (Markelova et al. 2000, Pouneva, 2000). The widely used ‘plate cell  
48 count’ consists of spreading a small volume of sample on a nutritive solid medium and  
49 counting the number of colonies formed after incubation (Allen and Smith 1966; Hwang and  
50 Horneland 1965; Madigan and Martinko 2006). The concentration of viable cells in the  
51 original sample can be determined by assuming that each visible colony originated from a  
52 single viable cell. Unfortunately, plate cell counts are often time-consuming: e.g. several days  
53 to weeks of incubation may be needed to detect colonies depending on the species studied.  
54 Alternatively, pulse amplitude magnitudes (PAM) measurements can give information on the  
55 physiological state of algal samples that can be correlated to viability in certain cases  
56 (Drábková et al. 2007). Another type of viability assessment relies on the use of dyes that  
57 stain preferentially living cells (Saga et al. 1987). For example, Fluorescein di-Acetate (FDA)

58 has been highly used in previous studies to assess microalgal viability (Berglund et al. 1987;  
59 Clarke et al. 2001; Steinberg et al. 2011). Once inside the cell, esterase from the living cells  
60 cleave the FDA molecule which results in the formation of fluorescein acetate that appears  
61 bright yellow-green under fluorescent light. Various dyes selectively stain dead cells only as  
62 they penetrate the damaged cytoplasmic membranes. Examples of dyes used in prior studies  
63 include visible dyes (methylene blue; Low et al. 1994, Evans blue; Saga et al. 1987, Direct  
64 brown; Venkata Mohan et al. 2008, etc.) and, more recently, fluorescent dyes such as SYTOX  
65 Green that becomes fluorescent once in contact with DNA (Breeuwer and Abe 2000). By  
66 counting the stained cells by microscopy, the viability of the sample can be estimated.  
67 Unfortunately, while staining techniques are faster than the plate cell count, they usually  
68 involve microscope counting which is time-consuming. Cells stained by fluorescent dyes can  
69 also be counted by fluorocytometry but microbiology labs are not systematically equipped  
70 with fluorocytometers due to the high cost of this type of equipment. In order to avoid using  
71 costly equipment, Capasso et al. (2003) developed a new viability technique based on  
72 spectrophotometric measurements. The authors used 3-(4,5-dimethylthiazol-2-yl)-5-(3-  
73 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (referred to as 'MTS') as this dye is  
74 degraded by viable cells into a colored compound ('formazan'). The authors proved that the  
75 color development due to the formazan production was proportional to the number of viable  
76 cells. However, it is difficult to accurately estimate the fraction of viable cells with this  
77 technique because productivity may also vary with the physiological state of the viable algae  
78 (i.e. the test could underestimate true viability if viable cells under stress produce less  
79 formazan than unstressed viable cells).

80

81 This study describes the colorimetric assay of viability for algae (CAVA) which is based on  
82 the selective adsorption of erythrosine by dead algal cells and only involves

83 spectrophotometric measurements. It has been experimentally shown that erythrosine was  
84 adsorbed by non-viable cells of 30 different algal strains (Markelova et al. 2000). Erythrosine  
85 was therefore selected as a 'staining dye' in this study. *Chlamydomonas reinhardtii* and  
86 *Chlorella vulgaris* were used as model strains representative of species broadly studied in  
87 algal biotechnology and with good potential for commercial applications (James et al. 2011;  
88 Mata et al. 2010; Spolaore et al. 2006).

89

## 90 2. Materials and methods

### 91 2.1. Algal strain and culture conditions

92 Axenic *C. reinhardtii* (6145C; Nelson Cawthron Institute, New Zealand) was cultivated in  
93 TAP medium (Gorman and Levine, 1969) with ammonium replaced by nitrate at the same  
94 nitrogen molar concentration. Axenic *C. vulgaris* (isolated in New Zealand by Landcare  
95 Research, Lincoln, New Zealand, GenBank *rbcL* sequence: EF589154) was cultivated in  
96 sterilized BG-11 medium (Andersen et al. 2005) enriched with nitrogen (0.25 g N-NO<sub>3</sub><sup>-</sup> L<sup>-1</sup>)  
97 and pH-buffered with phosphate salts (KHPO<sub>4</sub>: 1.52 g L<sup>-1</sup>; K<sub>2</sub>HPO<sub>4</sub>: 3.1 g L<sup>-1</sup>). Following  
98 inoculation (from either solid or liquid axenic cultures) in aseptic 250 mL Erlenmeyer flasks,  
99 algal cultures were incubated under constant conditions of temperature (25°C), agitation  
100 (orbital shaking at 160 rpm), atmospheric CO<sub>2</sub> partial pressure (0.5%), and illumination (14  
101 W m<sup>-2</sup> as photosynthetically active radiation at the culture surface). Cultures in the light-  
102 limited growth phase (linear growth) were used for the viability tests.

103

## 104 2.2. Dry weight (DW) measurements

105 A known volume of algae was filtered onto a pre-weighted filter (Membrane filters, 0.45  $\mu\text{m}$ ,  
106 47mm, MF-Millipore™, Merck Millipore, Billerica, MA, USA). The filter was then rinsed  
107 with reverse osmosis water in order to remove any salt from the filter and then dried at 105°C  
108 for one hour before being weighed. The DW concentration (expressed in the following in g  
109  $\text{DW L}^{-1}$ ) was then calculated by dividing the difference in weight by the volume of sample  
110 filtered. The DW concentration was measured in duplicates.

111

## 112 2.3. Isotherm characterization

113 Algal cells were centrifuged and re-suspended in phosphate-buffered saline solution (PBS;  
114 8.01 g  $\text{L}^{-1}$  NaCl; 0.20 g  $\text{L}^{-1}$  KCl; 1.60 g  $\text{L}^{-1}$   $\text{Na}_2\text{HPO}_4$ ,  $\text{H}_2\text{O}$ ; 0.27 g  $\text{L}^{-1}$   $\text{H}_2\text{PO}_4$ ). PBS was used  
115 to maintain identical conditions of temperature and pH conditions in all the assays and did not  
116 affect cell viability (data not shown). The algae were then heated at 60°C for 25 min before  
117 being cooled down to 20°C. The amount of erythrosine adsorbed on algae was found to  
118 stabilize after 25 min heating (see Online Resource S1). The solution of presumably killed  
119 cells was then diluted 2-fold and 4-fold to obtain a total of three algal solutions having  
120 different concentrations. Each of these solutions was exposed for 10 min to eight erythrosine  
121 concentrations (Erythrosin extra bluish, 1623-68-0 Sigma-Aldrich, USA) varying between  
122 0.004 to 0.12 g  $\text{L}^{-1}$ . The amount of erythrosine adsorbed onto the biomass and the  
123 concentration of free erythrosine left in solution were then determined by spectrophotometry  
124 (see Section 2.4) and found to stabilize within 5 min of exposure (see Online Resource S1). In  
125 the subsequent experiments, 10 min exposure was therefore applied in order to determine  
126 sorption properties at equilibrium.

127

128 The Langmuir and Freundlich isotherms were tested in order to characterize the sorption of  
129 erythrosine by dead cells. Several microalgal concentrations were tested. The Langmuir  
130 isotherm is described as:

$$131 \quad q = q_m \frac{C_w}{K + C_w} \quad (1)$$

132 where  $C_w$  is the erythrosine concentration in the supernatant ( $\text{g L}^{-1}$ ),  $q$  is the specific amount  
133 of erythrosine adsorbed ( $\text{g g DW}^{-1}$ ),  $q_m$  is the maximum specific amount of erythrosine  
134 adsorbed by dead cells ( $\text{g g DW}^{-1}$ ), and  $K$  ( $\text{g L}^{-1}$ ) is a saturation constant. The Freundlich  
135 isotherm is described as (Gupta et al. 2006):

$$136 \quad q = K_f (C_w)^n \quad (2)$$

137 where  $n$ , and  $K_f$  ( $\text{g}^{1-n} \cdot \text{L}^n \text{ g DW}^{-1}$ ) are constant parameters.

138

#### 139 2.4. Viability assay

140 In order to perform the CAVA test, algae were first centrifuged and re-suspended in PBS  
141 (Figure 1). A first aliquot of re-suspended algal solution (0.6 mL) was poured in an Eppendorf  
142 tube and heated for 25 min at  $60^\circ\text{C}$  in order to completely kill the algae. This solution was  
143 then cooled down to  $20^\circ\text{C}$  by soaking the tubes in a water bath and is henceforth referred to as  
144 the ‘Killed sample’. A second 0.6 mL aliquot, henceforth referred to as the ‘Sample’, was  
145 transferred in another Eppendorf tube and kept at  $20^\circ\text{C}$ . A third 0.6 mL aliquot of PBS was  
146 used as ‘Blank’. Following the preparation of these three solutions, 0.4 mL of a  $0.1 \text{ g L}^{-1}$   
147 erythrosine solution (in reverse osmosis water) at  $20^\circ\text{C}$  was added to each solution to reach an  
148 initial erythrosine concentration of  $0.04 \text{ g L}^{-1}$ . The three solutions were then maintained at  
149  $20^\circ\text{C}$  for 10 min. The tubes containing the Sample and the Killed sample were finally  
150 centrifuged at  $14,100 \text{ g}$  for 5 min. The erythrosine concentrations in the supernatants of the



151 three samples were measured by spectrophotometry at 526 nm (the maximum peak of light  
152 absorption by erythrosine solutions, determined by spectrophotometry). The optical densities  
153 were measured in duplicate for the Sample and the Killed Sample and in triplicate for the  
154 Blank (cuvettes: Thermo Fischer Scientific Cuvette 10x10x45mm polystyrene;  
155 Spectrophotometer: Shimadzu UV-1800). Erythrosine concentration  $C_w$  was found to be  
156 proportional to the optical density at 526 nm ( $OD_{526}$ ) according to the equation:

$$157 \quad OD_{526} = \alpha C_w \quad (3)$$

158 where the coefficient  $\alpha$  was experimentally determined as  $86.6 \text{ L g}^{-1}$  ( $R^2 = 0.9996$ ,  $N = 8$ ).

159

## 160 2.5. Expression of the viability

161 The viability of an algal sample was determined from the optical densities of the supernatant  
162 the Killed sample ( $OD_K$ ), the Sample ( $OD_S$ ), and the Blank ( $OD_B$ ). The following assumptions  
163 were made:

- 164 1. Only dead cells adsorb erythrosine;
- 165 2. The sorption of erythrosine onto dead algal cells can be described by a Langmuir isotherm;
- 166 3. The characteristic constants of erythrosine sorption ( $q_m$  and  $K$  in Equation 1) are not  
167 affected by the mechanism causing cellular death;
- 168 4. Erythrosine losses by degradation and volatilization are negligible.

169 The validation of Assumptions 1 to 3 is discussed thereafter in the results and discussion  
170 section. Preliminary kinetic studies (Online Resource, S1) validated the Assumption 4.

171

172 Assuming there is no loss of erythrosine, a mass balance yields:

$$173 \quad Q_n = Q_{a,n} + Q_{w,n} \quad (4)$$

174 where  $Q_n$  (g) is the amount of erythrosine initially added in the tube,  $Q_{a,n}$  (g) is the amount of  
 175 erythrosine adsorbed by the dead algae,  $Q_{w,n}$  (g) is the amount of free erythrosine remaining in  
 176 the solution, and  $n$  stands for the solution analyzed (K – Killed sample, S – Sample and B-  
 177 Blank). As the same amount of erythrosine is initially added in each solution,  $Q_n$  can be  
 178 determined as follows:

$$179 \quad Q_K = Q_S = Q_B = C_{w,B}V \quad (5)$$

180 where  $C_{w,B}$  (g L<sup>-1</sup>) is the concentration of free Erythrosine in the Blank and  $V$  is the volume of  
 181 the solution contained in the tube (L). Assuming that only dead cells adsorb erythrosine,  
 182 Equation 4 applied to the Sample and the Killed sample can be written as follows:

$$183 \quad Q_{a,n} + Q_{w,n} = q_n X_{d,n} V + C_{w,n} V \quad (6)$$

184 where  $q_n$  is the specific amount of erythrosine adsorbed by algae (in g g DW<sup>-1</sup>),  $X_{d,n}$  the dead  
 185 cell concentration (in g DW L<sup>-1</sup>),  $V$  the volume of the solution (L), and  $C_{w,n}$  the concentration  
 186 of erythrosine remaining in the solution. In the case of the Killed Sample, the dead cell  
 187 concentration equals the total cell concentration ( $X_T$ , g DW L<sup>-1</sup>) and, assuming that  
 188 erythrosine sorption is described by a Langmuir isotherm, Equation 6 becomes:

$$189 \quad Q_{a,K} + Q_{w,K} = q_{m,H} \frac{C_{w,K}}{C_{w,K} + K_H} X_T V + C_{w,K} V \quad (7a)$$

190 When applied to the Sample, Equation 6 becomes:

$$191 \quad Q_{a,S} + Q_{w,S} = q_{m,NH} \frac{C_{w,S}}{C_{w,S} + K_{NH}} X_d V + C_{w,S} V \quad (7b)$$

192 where  $q_{m,H}$  ( $q_{m,NH}$ ) and  $K_H$  ( $K_{NH}$ ) are the maximum specific amount of erythrosine adsorbed by  
 193 the dead cells (g g DW<sup>-1</sup>) and the Langmuir's saturation constant (g L<sup>-1</sup>) for the heated (H)  
 194 (and non-heated; NH) cells, and  $X_d$  is the dead cell concentration in the sample (g DW L<sup>-1</sup>).  
 195 Combining Equations 3, 5, 7a, and 7b yield the two following equations:

196 
$$X_T = \frac{1}{q_{m,H}\alpha} \frac{K_H' + OD_K}{OD_K} (OD_B - OD_K) \quad (8a)$$

197 
$$X_d = \frac{1}{q_{m,NH}\alpha} \frac{K_{NH}' + OD_S}{OD_S} (OD_B - OD_S) \quad (8b)$$

198 where  $K_H'$  and  $K_{NH}'$  are the products of  $\alpha$  and  $K_H$  and  $K_{NH}$ , respectively. The viability  $\nu$  is  
 199 derived from the ratio of the concentration of dead cells,  $X_d$ , over the total cell concentration  
 200  $X_T$ :

201 
$$\nu = 1 - \frac{X_d}{X_T} = 1 - \frac{q_{m,H}}{q_{m,NH}} \frac{OD_K}{OD_S} \frac{K_{NH}' + OD_S}{K_H' + OD_K} \frac{OD_B - OD_S}{OD_B - OD_K} \quad (9)$$

202 Assuming the values of  $q_m$  and  $K$  are not affected by the mechanism causing cellular death  
 203 (Assumption 3), the viability can be expressed as follows:

204 
$$\nu = 1 - \frac{OD_K}{OD_S} \frac{K' + OD_S}{K' + OD_K} \frac{OD_B - OD_S}{OD_B - OD_K} \quad (10)$$

205 where  $K'$  is the product of  $\alpha$  and  $K_H$  (or  $K_{NH}$  assuming they are equal).

206 **The viability of microalgae cultures exposed to different stresses and in different**  
 207 **physiological states can be measured with this technique. In fact, comparing the quantity of**  
 208 **erythrosine adsorbed by the Sample to the quantity of erythrosine adsorbed by the Killed**  
 209 **Sample (Equation 10) enables accounting for the potential variations of adsorption properties**  
 210 **of algal cells as long as Assumption 3 is verified.**

211

## 212 2.6. Comparison with the measurements of oxygen production rates

213 Aliquots of killed and viable cells (from the same initial culture, the 'killed' cells being heated  
 214 at 60°C for 25 min and cooled down at 20°C) were mixed at known volumetric ratios of 1:0;  
 215 3:1; 2:2; 1:3; and 0:1. The viability of the resulting five solutions was then assessed using the  
 216 CAVA test and short-term *in situ* oxygen productivity (in mg O<sub>2</sub> L<sup>-1</sup>s<sup>-1</sup>) was measured using

217 the device presented by Béchet et al. (2014). This device included six cylindrical vessels  
218 (diameter: 49.2 mm; height: 40 mm) equipped with dissolved oxygen electrodes (Model  
219 DO50-GS, Hach) and placed above LED lamps (12V PHILIPS Endura LED 10W MR16  
220 Dimmable 4000K). The sides and the top lid of the vessels were opaque and the bottom wall  
221 was made of a transparent polycarbonate plastic. A complete description of the device can be  
222 found in Béchet et al. (2014). The five algal solutions placed in the vessels of the device were  
223 exposed to the same incident light intensity ( $63 \pm 7 \text{ W m}^{-2}$  as photosynthetically active  
224 radiation) and the temperature in each vessel was maintained at  $22^\circ\text{C}$ . Dissolved oxygen  
225 concentration was recorded over time during the light and dark phases. The net oxygen  
226 productivity and the respiration rate were obtained by linear regression. The respiration rates  
227 in the dark and in the light phase were assumed to be equal. As a result, the gross rate of  
228 oxygen production in each vessel was obtained by adding the rate of respiration (in absolute  
229 values) to the rate of net oxygen production as algae respire in the light phase.

230

231 2.7. Application of the CAVA test in the case of death by UV-irradiation and  $\text{H}_2\text{O}_2$  exposure

232 The CAVA test was applied to monitor changes in cell viability in algal cultures exposed to  
233 UV-irradiation or hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). For UV-irradiation, an algal sample was  
234 separated in solutions of around 150 mL placed in four different 1L-beakers. Two beakers  
235 were irradiated by a UV lamp (Philips TUV 30W/G30 T8) placed approximately 20 cm above  
236 the sample (UV-light intensity:  $7.8 \text{ W m}^{-2}$ ; ILT 1400 radiometer photometer equipped with a  
237 255 nm filter) while the two other beakers were protected from UV light by an opaque cover.  
238 The four beakers (two sets of two replicates) were placed in an orbital shaker (agitation at 150  
239 rpm) to provide well-mixed conditions (no sedimentation was observed). Water evaporation  
240 was compensated for by weighing the beakers and adding reverse-osmosis water every day in

241 each beaker. The changes in viability in the four solutions were monitored daily. For H<sub>2</sub>O<sub>2</sub>  
242 exposure, H<sub>2</sub>O<sub>2</sub> (30% v/v; Code: H/1800/15, Fisher Scientific UK, Bishop Meadow Road,  
243 Loughborough) was added to algal cultures (volume ratio H<sub>2</sub>O<sub>2</sub> (30%): algal culture of 1:4 or  
244 1:9, see Figure 5 for details) in a closed Duran bottle continuously mixed by magnetic  
245 stirring. Samples were withdrawn at regular intervals, centrifuged and re-suspended twice in  
246 PBS in order to avoid any degradation of erythrosine by remaining H<sub>2</sub>O<sub>2</sub>. CAVA tests were  
247 performed on the re-suspended solutions by following the protocol described in Section 2.4.  
248

## 249 2.8. Monte-Carlo simulations for uncertainty estimation

250 Uncertainty on viability measurements was introduced by experimental error on the optical  
251 densities measurement ( $OD_S$ ,  $OD_K$  and  $OD_B$  in Equation 10). A statistical analysis on  
252 duplicate measurements revealed that the error on optical density was normally distributed  
253 (see Online Resource S2 for details). Monte-Carlo simulations were then used in order to  
254 determine how the error on experimental measurements impacted the accuracy of the viability  
255 estimations as described in Online Resource S2.

256

## 257 3. Results and Discussion

### 258 3.1. Characterization of the adsorption isotherm

259 Erythrosine absorption onto biological tissues has been shown to be characterized by both the  
260 Langmuir and the Freundlich isotherm for hen feathers (Gupta et al. 2006), de-oiled mustard  
261 (Jain and Sikarwar 2009), or de-oiled soya (Mittal et al. 2006). However, and to the best of  
262 our knowledge, the adsorption of erythrosine by dead algal cells has never been characterized  
263 and is the object of this first section.

264

265 According to Figure 2, the Freundlich isotherm could not represent erythrosine adsorption  
266 onto killed *C. reinhardtii* cells. On the other hand, both Langmuir and Freundlich isotherms  
267 could describe the experimental data collected for *C. vulgaris* (Figure 2). The Langmuir  
268 isotherm was shown to describe the sorption equilibrium over a large range of algal  
269 concentrations (0.24-0.96 g DW L<sup>-1</sup> for *C. reinhardtii* and 0.43-1.7 g DW L<sup>-1</sup> for *C. vulgaris*)  
270 and erythrosine concentrations (0.01-0.12 g L<sup>-1</sup>). In addition, the parameters of the Langmuir  
271 isotherm,  $q_m$  and  $K$ , (Equation 1) did not significantly change with the algal concentrations as  
272 shown by the confidence intervals in Table 1. Langmuir's behavior was also observed when  
273 *C. vulgaris* cells were killed by exposure to hydrogen peroxide instead of heating (data not  
274 shown). High temperature is known to kill cells by protein denaturation and hydrogen  
275 peroxide is a strong oxidizer, affecting, for instance, enzymes in the Calvin Cycle (Drábková  
276 et al. 2007; Takeda et al. 1998). **These results validate Assumption 2 (see Section 2.5) and the**  
277 **Langmuir model was subsequently used as the basis for calculating viability. In Sections 3.2**  
278 **to 3.4, the half-saturation constants of the Langmuir isotherm (and the associated confidence**  
279 **interval) were taken equal to the values resulting from the fitting of the experimental points**  
280 **obtained for the three algal concentrations (values in bold in Table 1). The Langmuir isotherm**  
281 **suggests the binding of erythrosine as a monolayer. Differences in the cell wall biochemistry**  
282 **of the two algal species studied likely explain the difference of  $q_m$  values found between these**  
283 **species (Table 1). Almost no erythrosine was adsorbed by non-killed cells, suggesting that the**  
284 **adsorption of erythrosine onto dead cells is likely associated with a loss of cell wall integrity**  
285 **causing binding onto otherwise unavailable intracellular material (Markelova et al. 2000).**

286

### 287 3.2. Comparison with oxygen productivity

288 In order to benchmark the CAVA test against other viability assays, oxygen productivity and  
289 colorimetric viability were measured in partly killed algal cultures. As can be seen in Figure  
290 3, oxygen productivity was close to linearly correlated to the measured viability in algal  
291 samples containing different fractions of dead and viable cells, the uncertainty being mostly  
292 caused by experimental error. Because oxygen production by algae is the result of  
293 photosynthesis and cannot be performed by dead cells, the good agreement between the  
294 CAVA results and oxygen productivities indirectly indicates that living cells do not adsorb  
295 erythrosine, thus validating Assumption 1 (see Section 2.5). A slight limitation of the  
296 technique is the fact that the measured viability in the Killed sample was estimated to be close  
297 to 10-15% (Figure 3). This limitation is further discussed in Section 3.4.

298

### 299 3.3. Application of the CAVA test in the case of death by UV-irradiation and H<sub>2</sub>O<sub>2</sub> exposure

300 Figures 4 and 5 show that the measured viability of cultures exposed to H<sub>2</sub>O<sub>2</sub> and UV light  
301 decreased over time, as expected. The decrease of viability in the cultures exposed to UV light  
302 was not due to natural death as the viability of the algal sample not exposed to UV light  
303 remained constant over the duration of the experiment (Figure 4). In addition, the CAVA tests  
304 showed that the rate of cellular death seemed to increase, as expected, with the H<sub>2</sub>O<sub>2</sub>  
305 concentration for *C. reinhardtii* (Figure 5). The expected decrease in viability observed when  
306 cells are exposure to UV light or hydrogen peroxide suggests that the CAVA test is able to  
307 monitor the viability of algal samples killed by different techniques. This result is important  
308 as heating, H<sub>2</sub>O<sub>2</sub> and UV light cause death through different mechanisms: heating denatures  
309 proteins; H<sub>2</sub>O<sub>2</sub> is a strong oxidizer, and UV light damages DNA. In spite of these differences,  
310 the CAVA test yielded trends that were consistent with expected results.

311

#### 312 3.4. Protocol optimization

313 Erythrosine concentration can be optimized to maximize the precision of the CAVA test. The  
314 accuracy of the measured viability depends on the amount of erythrosine adsorbed by algae.

315 This quantity is obtained by the difference between the amount of erythrosine initially  
316 introduced and the amount remaining the supernatant. As a result, the greater the amount of

317 erythrosine adsorbed, the higher the accuracy. It is therefore recommended to use

318 concentrated algal samples (e.g. > 1 g/L) to maximize the amount of erythrosine adsorbed by

319 dead cells when using this assay. For example, in a hypothetical case of a 50%-viable sample

320 of *C. vulgaris*, the confidence interval on the measured viability can decrease from ±20% to

321 ±6% when the algal concentration increases from 0.5 to 2 g DW L<sup>-1</sup> (see Online Resource S3

322 for details). Measuring the optical densities  $OD_S$  and  $OD_K$  in triplicates should reduce

323 uncertainty further.

324

325 A limitation of the CAVA test is that the measured viability of cultures exposed to H<sub>2</sub>O<sub>2</sub> did

326 not reach exactly 0% but stabilized at values close to 10% for *C. reinhardtii* and 15% for *C.*

327 *vulgaris* (Figure 5). These plateau values were reproducible, as shown in Figure 5, and

328 therefore unlikely caused by experimental error. However, this overestimation of viability

329 was not observed when algae were killed by UV-exposure. Microalgae exposure to H<sub>2</sub>O<sub>2</sub> may

330 change adsorption properties of the cells (i.e. values of  $K$  or  $q_m$ ), affecting the viability

331 calculation. As mathematically demonstrated in the Online Resource S4, the variability of  $K$

332 (due to physiological changes) does not significantly increase the uncertainty of the estimated

333 viability. However, the value of the coefficient  $q_m$  may differ between the Sample and the

334 Killed sample due to different combinations of stressors involved (e.g. the combined action of



335 H<sub>2</sub>O<sub>2</sub> exposure and heating in the Killed sample versus H<sub>2</sub>O<sub>2</sub> exposure only for the Sample).  
336 Such difference could explain the 10-15% overestimation of viability seen in Fig. 5. This  
337 explanation is consistent with the observations of Imase et al. (2013) who showed that H<sub>2</sub>O<sub>2</sub>  
338 may affect *Chlorella sorokiniana* by creating tiny holes in the cell membranes. This possible  
339 change of adsorption properties in the presence of H<sub>2</sub>O<sub>2</sub> challenges Assumption 3. In order to  
340 improve the method for viability determination when microalgae are subjected to hydrogen  
341 peroxide stress, further assays should be performed to characterize erythrosine adsorption on  
342 those cells.

343

344 Even if this study was limited to two algal species, erythrosine was shown to be adsorbed by  
345 more than 30 algal species including cyanobacteria by Markelova et al. (2000), suggesting  
346 that the CAVA test can potentially be applied to an important variety of algal species. In  
347 addition, the same protocol can theoretically be applied to different dyes such as Methylene  
348 blue (Low et al. 1994), Evans blue (Saga et al. 1987), or Direct brown (Venkata Mohan et al.  
349 2008) which were shown to be adsorbed by dead algal cells. The technique is also relatively  
350 fast compared to other viability assays and is also practical as no microscopic observation is  
351 required. For example, the dual fluorescence assay proposed by Sato et al. (2004) and Shulze  
352 et al. (2011) using SYTOX Green and auto-fluorescence requires microscopy counting to  
353 distinguish living cells (appearing red) from dead cells (appearing green). Counting could be  
354 easily performed using a fluorocytometer but this type of equipment is expensive. In contrast,  
355 the CAVA test only requires a common and affordable spectrophotometer. To the best of our  
356 knowledge, the CAVA test is the first spectrophotometric viability assay that enables the  
357 direct quantification of the fractions of living and dead cells. The viability method presented  
358 in this study has therefore the potential to offer a fast and universal approach to measure the  
359 viability of algal samples. Among its possible applications, the CAVA test may be used to

360 evaluate microalgal viability in outdoor cultivation systems where cells undergo stresses such  
361 as high temperature and UV-irradiation.

362

#### 363 4. Conclusions

364 **First**, erythrosine adsorption onto dead *C. vulgaris* and *C. reinhardtii* cells could be described  
365 by the Langmuir isotherm. **By applying the CAVA technique on partially killed samples**  
366 **containing both living and dead algal cells**, the CAVA results were in good agreement with  
367 *in-situ* measurements of oxygen productivity. **In addition**, the CAVA test could quantify cells  
368 viability **over time** in algal cultures subjected to diverse mechanisms causing cellular death  
369 (heating, UV-irradiance, exposure to H<sub>2</sub>O<sub>2</sub>) for the two algal species studied. **Finally**, the  
370 CAVA test can hypothetically be applied to any dye exclusively adsorbed by dead algal cells.  
371 The technique has therefore the potential to offer a fast, practical and universal approach to  
372 measure the viability of algal samples.

373

#### 374 5. Acknowledgments

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- 458

459 Figures

460

461 **Fig. 1** Schematic description of the protocol of the **colorimetric assay of viability for algae**

462 **(CAVA)**

463

464 **Fig. 2** Amount of erythrosine adsorbed by dead algal cells for different erythrosine

465 concentrations **in the supernatant at equilibrium**. Algal concentrations (*C. reinhardtii*/*C.*

466 *vulgaris* in g DW L<sup>-1</sup>: red circles: 1.7/0.96; blue diamonds: 0.85/0.48; black crosses:

467 0.43/0.24). Plain line: fitting with a Langmuir isotherm; **dashed**-line: fitting with a Freundlich

468 isotherm. Values of fitted parameters are given in Table 1. Error bars represent 95%

469 confidence intervals calculated using Monte-Carlo simulations

470

471 **Fig. 3** Comparison between the oxygen productivity and the **measured** viability of algal

472 samples partly killed by heating (**a**: *C. reinhardtii*, 0.32 g DW L<sup>-1</sup>; **b**: *C. vulgaris*, 0.32 g DW

473 L<sup>-1</sup>). The oxygen productivity was normalized to the oxygen productivity in the sample

474 containing only non-killed cells. The error bars represent the 95% confidence intervals

475 estimated using Monte-Carlo simulations

476

477 **Fig. 4** Viability of cultures exposed to UV light over time. Algal concentrations: *C.*

478 *reinhardtii*: 0.57 g DW L<sup>-1</sup>; *C. vulgaris*: 0.69 g DW L<sup>-1</sup>. The error bars represent 95%

479 confidence intervals calculated through Monte-Carlo simulations

480

481 **Fig. 5** Viability of cultures exposed to **hydrogen peroxide**. H<sub>2</sub>O<sub>2</sub> concentration/algal

482 concentration: *C. reinhardtii*: white: 6% / 0.78 g DW L<sup>-1</sup>; grey: 3% / 0.88 g DW L<sup>-1</sup>; *C.*

483 *vulgaris*: white: 6% / 1.3 g DW L<sup>-1</sup>; grey: 6% / 0.48 g DW L<sup>-1</sup>. The error bars represent the  
484 95% confidence interval calculated through Monte-Carlo simulations



**Table 1:** Langmuir and Freundlich isotherms parameters for *C. reinhardtii* and *C. vulgaris*

( $X$ : algal concentration in g DW/L; units:  $q_m$ : mg/g;  $K$ : mg/L;  $K_f$ :  $10^{-3} \text{g}^{1-n} \cdot \text{L}^n / \text{g}^n$ ;  $n$ : -). 95 % confidence intervals calculated using Monte-Carlo simulations are shown in parenthesis.

Values in bold characters result from the fitting of data from all algal concentrations.

Algae	$X$	Langmuir isotherm				Freundlich isotherm			
		$q_m$	$K$	$q_m$	$K$	$K_f$	$n$	$K_f$	$n$
<i>C. reinhardtii</i>	0.96	84.6 (2.9)	21.4 (2.0)			418 (170)	0.623 (0.114)		
	0.48	91.4 (5.0)	27.2 (4.0)	<b>86.4</b> <b>(4.1)</b>	<b>25.0</b> <b>(3.1)</b>	403 (231)	0.632 (0.128)	<b>366</b> <b>(121)</b>	<b>0.614</b> <b>(0.090)</b>
	0.24	86.3 (10.2)	29.0 (9.2)			387 (754)	0.626 (0.243)		
<i>C. vulgaris</i>	1.7	17.8 (2.2)	25.3 (7.1)			53.6 (16.2)	0.516 (0.087)		
	0.85	25.1 (6.9)	44.9 (24.2)	<b>22.4</b> <b>(4.7)</b>	<b>37.8</b> <b>(15.8)</b>	64.8 (40.5)	0.540 (0.151)	<b>58.6</b> <b>(21.7)</b>	<b>0.529</b> <b>(0.103)</b>
	0.43	25.7 (76.5)	48.7 (364)			70.8 (681.4)	0.532 (0.292)		

Figure 1  
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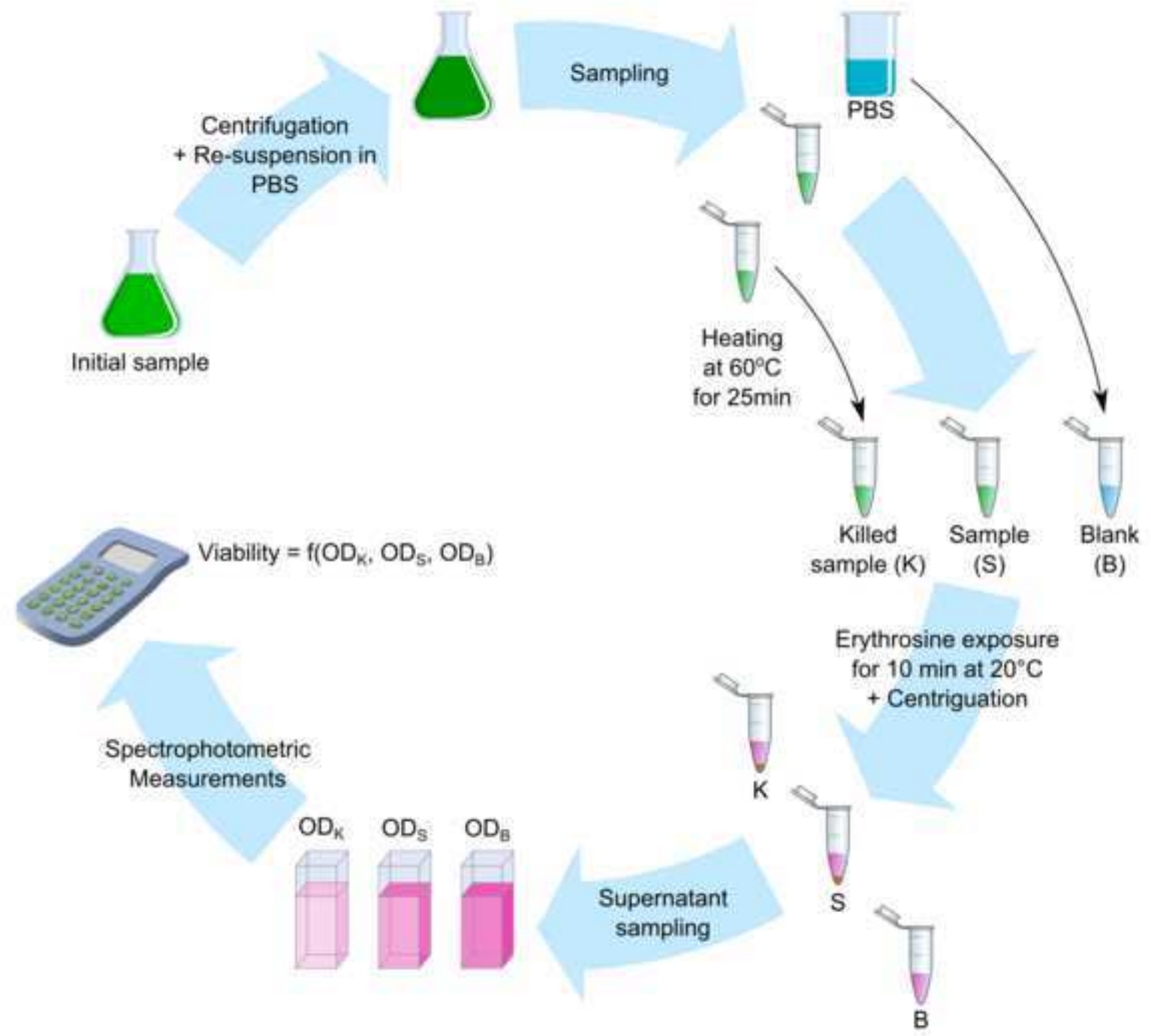


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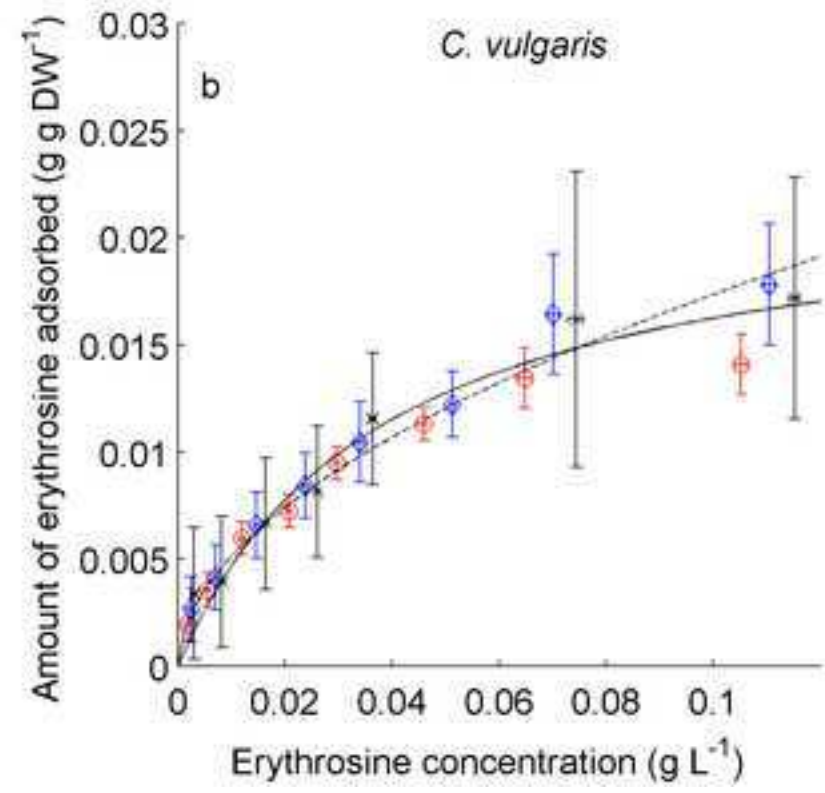
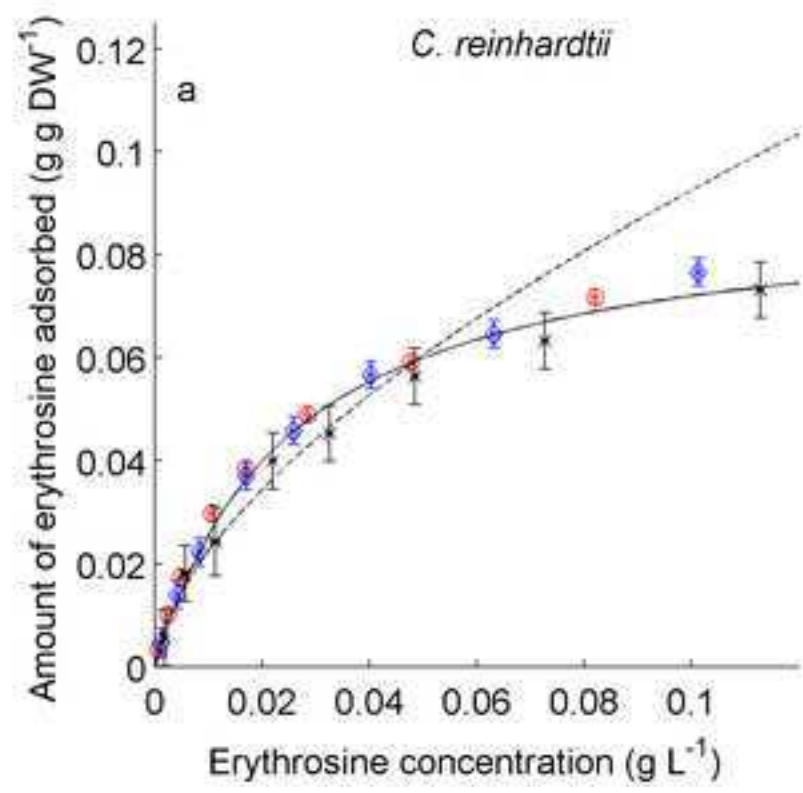


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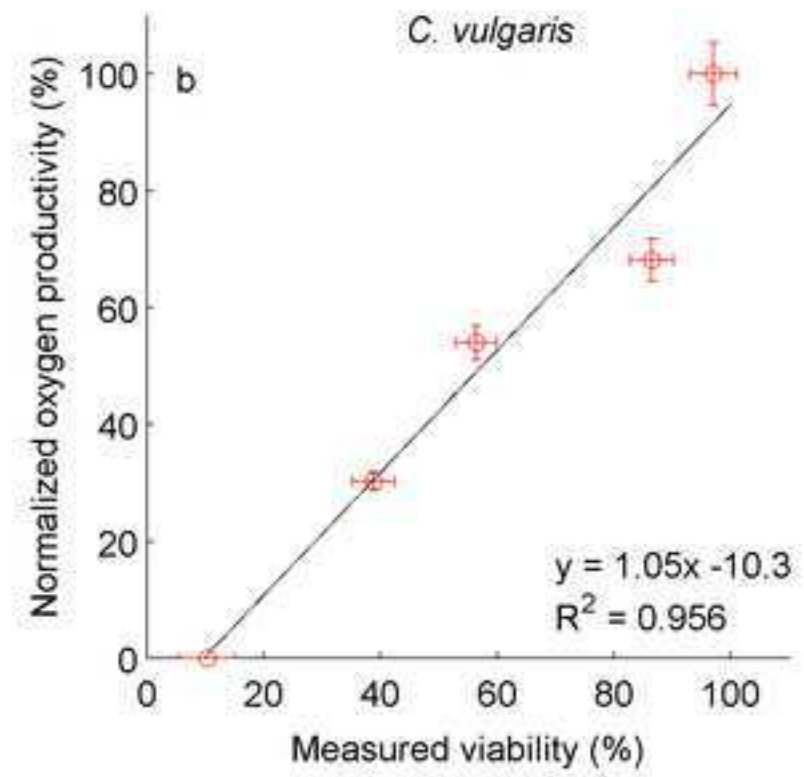
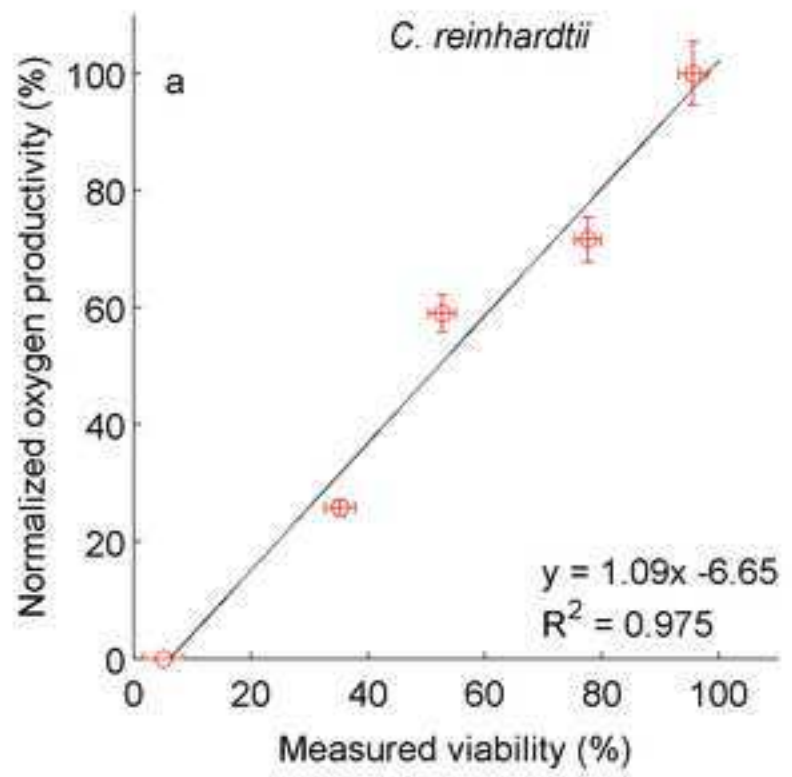


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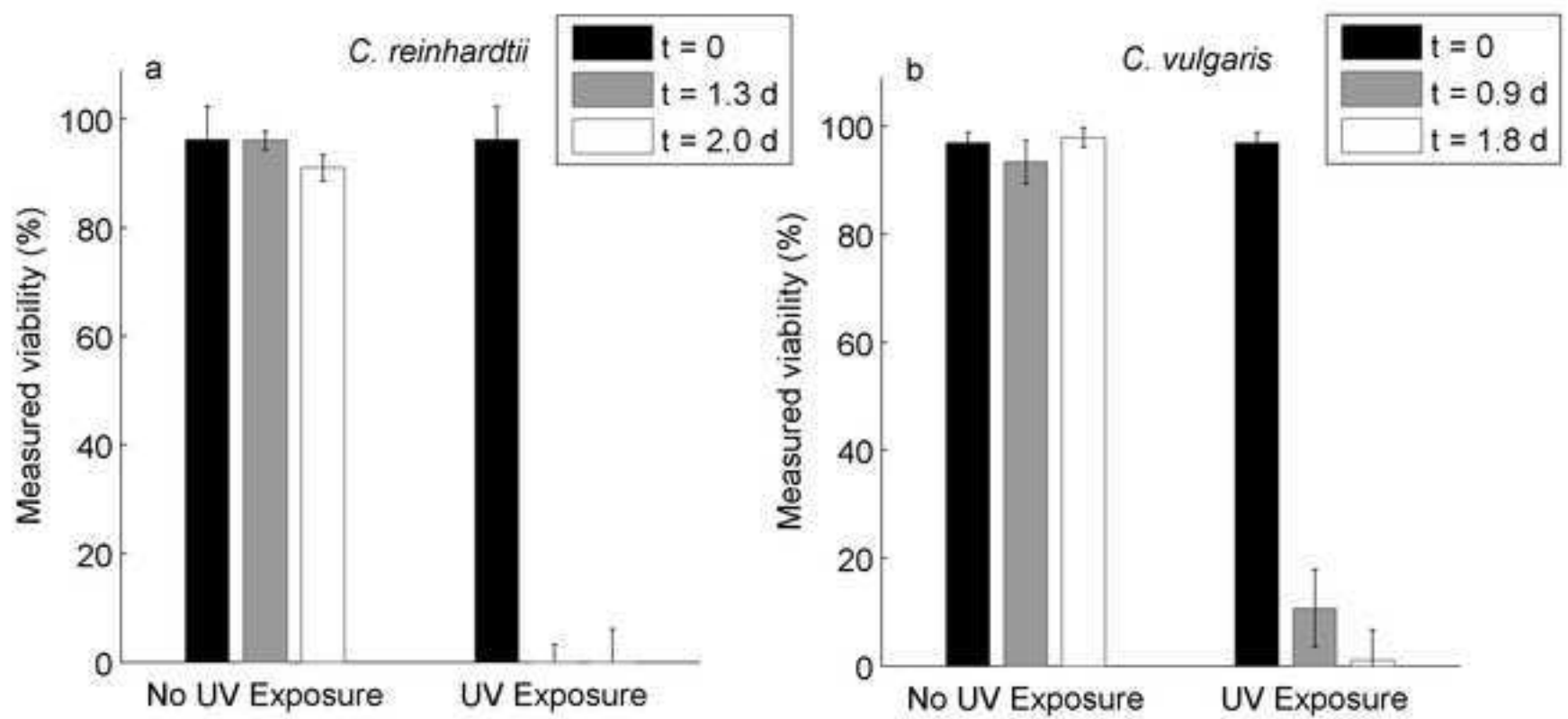
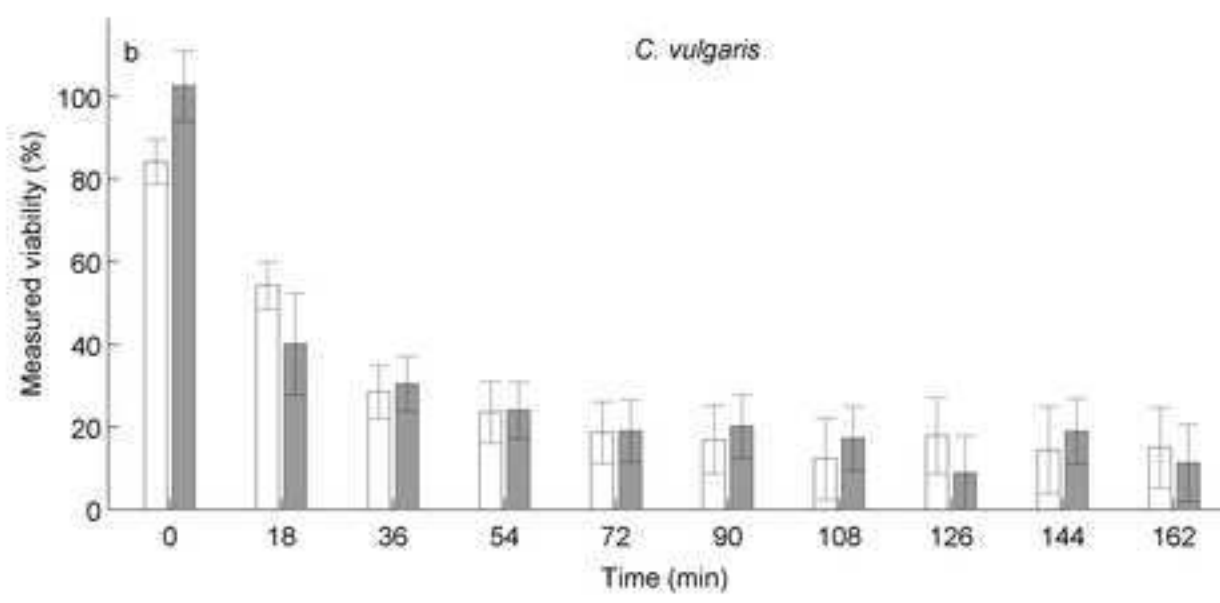
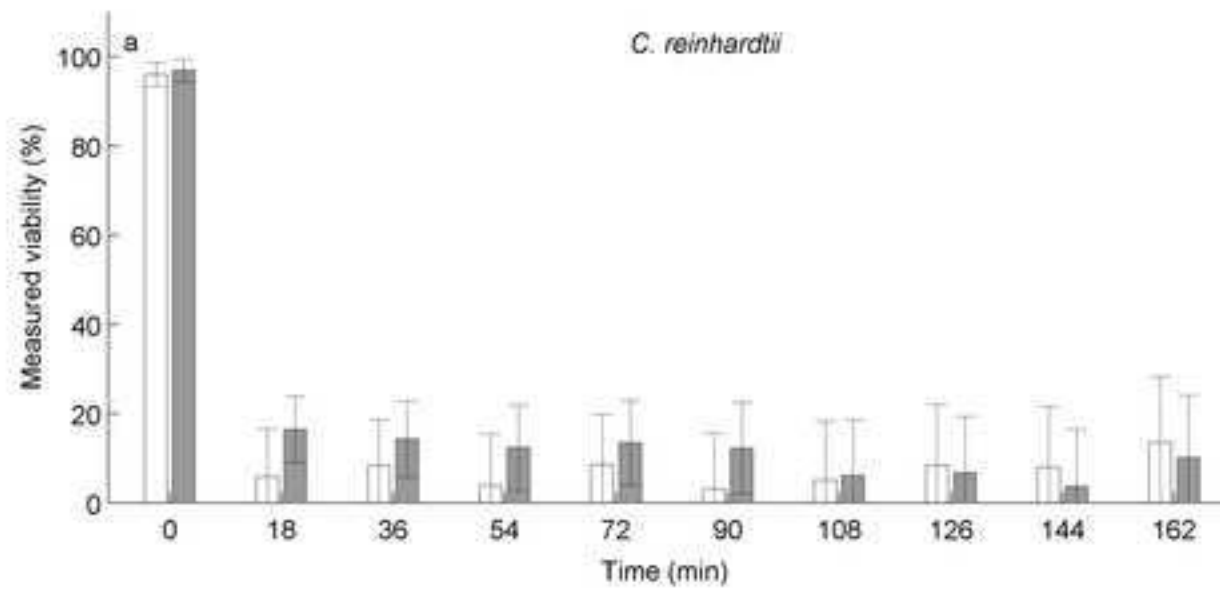


Figure 5

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# The colorimetric assay of viability for algae (CAVA): a fast and accurate technique – Supplementary information

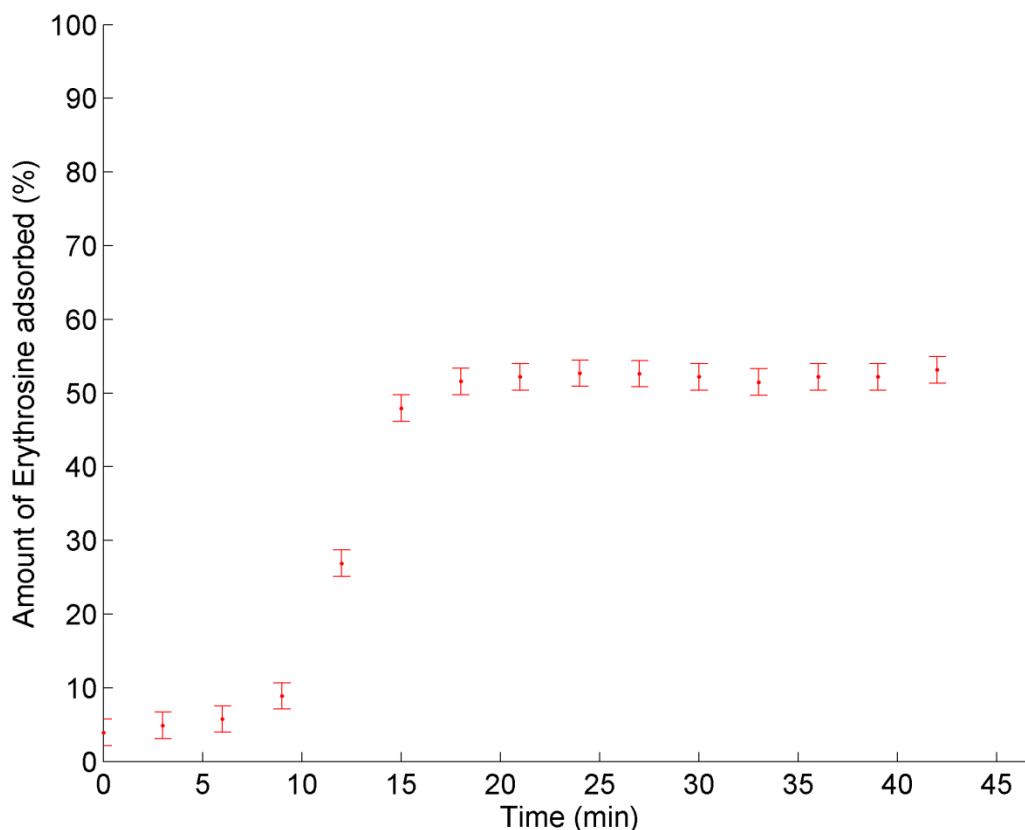
Quentin Béchet, Ivan Feurgard, Benoit Guieysse, Filipa Lopes

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# S1: Kinetic studies

## 1.1. Impact of heating time on the amount of erythrosine adsorbed by killed algae

A sample of *Chlorella vulgaris* was heated for different periods of time at 60°C. The cells were then exposed to erythrosine for 10 minutes (initial concentration of 0.04 g L<sup>-1</sup>) and the amount of erythrosine adsorbed by the cells was determined by colorimetry as described in the main manuscript. Figure S1-1 shows that the amount of erythrosine adsorbed by *C. vulgaris* cells stabilized after approximately 20 minutes of heating (The fact that 5% of erythrosine was adsorbed at t=0 indicates that the sample tested was not entirely viable before the heating). For this reason, the Killed sample in the CAVA test was heated for 25 minutes.

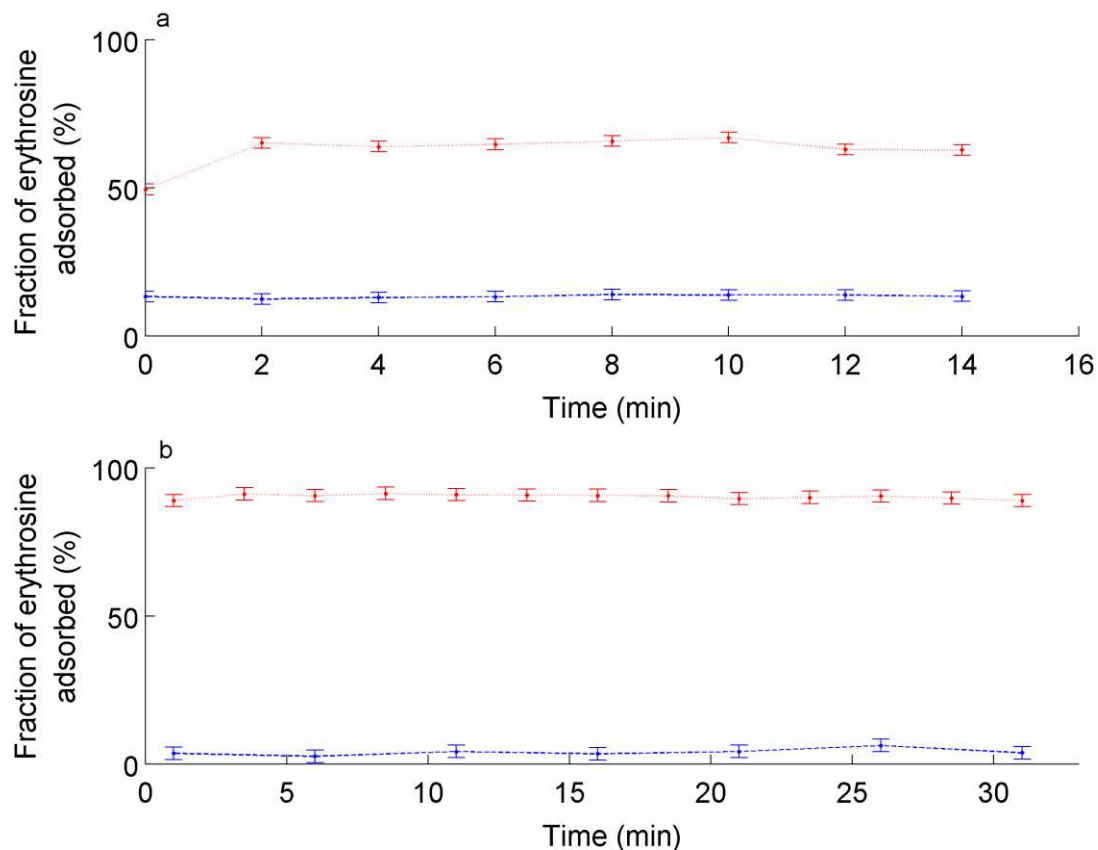


**Figure S1-1:** Evolution of the amount of erythrosine adsorbed by a *C. vulgaris* sample with the time of heating at 60°C. The error bars represent the confidence interval at 95% calculated through Monte-Carlo simulations as discussed in S2.



## 1.2. Kinetic of erythrosine adsorption by killed cells

Killed samples of *C. vulgaris* were exposed to erythrosine (initial concentration  $0.04 \text{ g L}^{-1}$ ) for different periods of time. In parallel, a non-killed sample originating from the same algae sample was exposed to the same dose of erythrosine. The amount of erythrosine adsorbed by the samples was estimated by following the protocol described in the main manuscript. Figure S1-2 shows that the equilibrium between the solution and the algae was reached within less than 5 minutes exposure to erythrosine. In addition, as the amount of erythrosine adsorbed by the non-heated cells was constant over time, this indicates that erythrosine was not degraded through a chemical reaction or a biological mechanism, confirming then the Assumption 4 of the CAVA test presented in the main manuscript.



**Figure S1-2:** Evolution of the fraction of erythrosine adsorbed by *C. vulgaris* samples exposed to 70°C for 25 minutes (a) and to 95°C for 10 minutes (b). Red point line: killed sample; blue dashed-line: non-killed sample. The error bars represent the 95% confidence interval estimated using Monte-Carlo simulations as described in S2.

## S2: Estimation of confidence intervals using Monte-Carlo simulations

### 2.1. Introduction

All the measurements of optical densities **for the Sample and the Killed sample ( $OD_S$  and  $OD_K$ )** in the protocol of the CAVA test described in the main manuscript were made in duplicates, representing a total of 684 duplicates. The objective of this supplementary information is to explain how these duplicate measurements were used in a statistical analysis to estimate a confidence interval on the viability measurements.

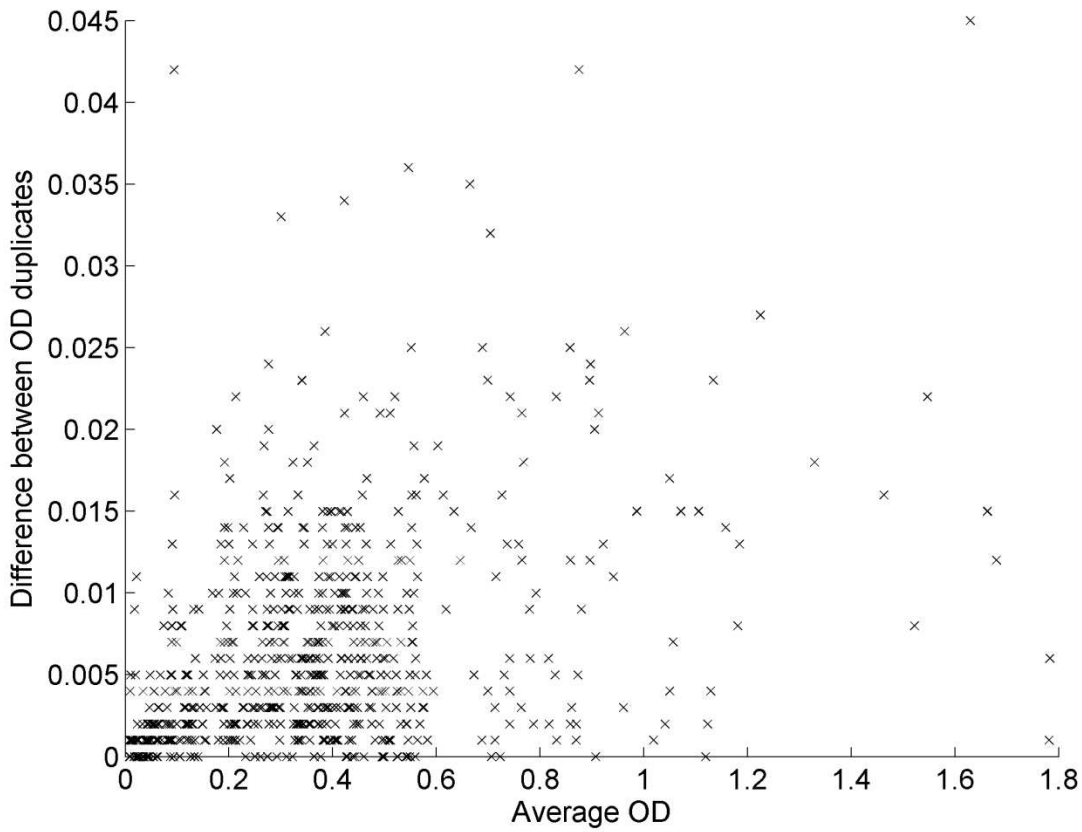
### 2.2. Uncertainty on the optical density measurements

Figure S2-1 shows that the difference between two OD duplicates is not clearly correlated to the concentration of erythrosine in the cuvette (represented by the average OD). As a result, the difference between duplicates is likely to be due to factors other than the algal concentration such as **pipetting errors** or defaults of the cuvette (scratch, dust, etc.)

By assuming that the error on an OD measurement follows a normal distribution centered on 0 and characterized by a standard deviation  $\sigma$ , the difference between two duplicates should follow a normal distribution also centered on 0 but characterized by a standard deviation  $\sqrt{2}\sigma$ . Consequently, the absolute value of this difference follows the half-normal distribution, represented by the following probability density function (Bland, 2005):

$$f(x) = \frac{1}{\sigma\sqrt{\pi}} \exp\left(-\frac{x^2}{4\sigma^2}\right) \text{ for } x \geq 0 \quad (\text{S2-1a})$$

$$f(x) = 0 \text{ for } x < 0 \quad (\text{S2-1b})$$



**Figure S2-1:** Difference between two OD duplicates as a function of the average OD in the cuvette.

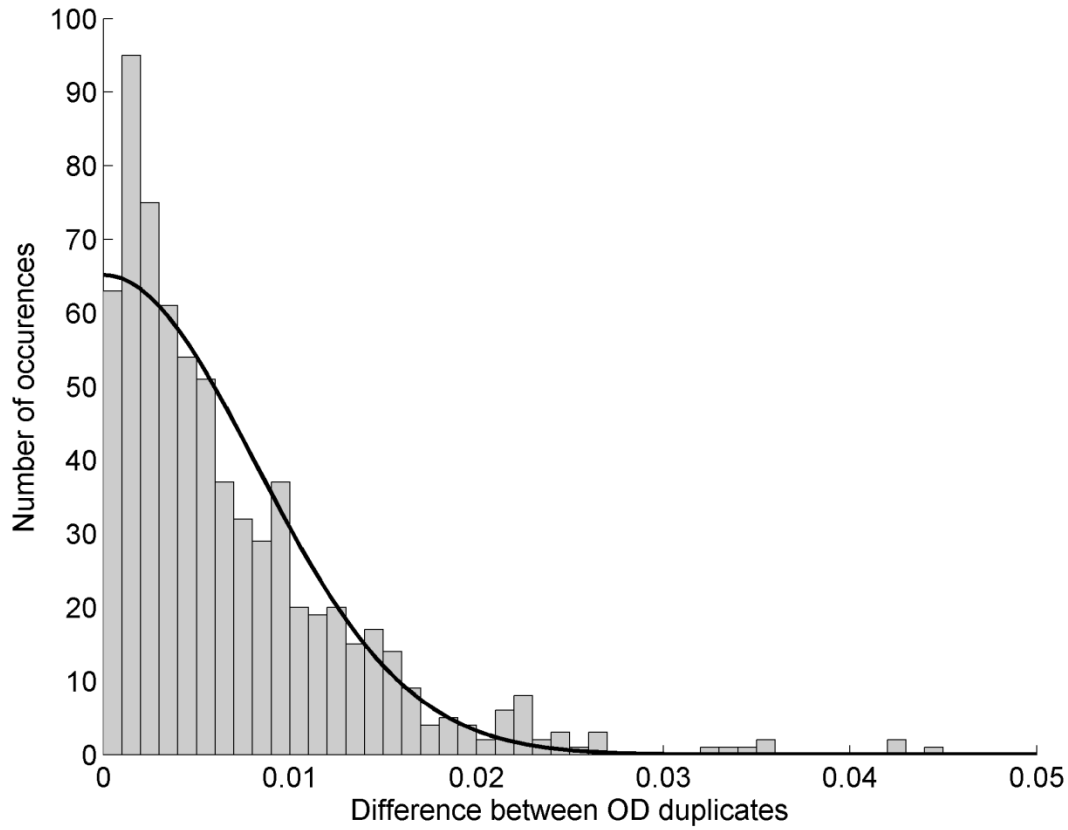
Figure S2-2 shows that the absolute value of the difference between OD duplicates indeed follows a half-normal distribution. As demonstrated by Bland (2005), the expectation of a variable  $X$  obeying a half-normal distribution is:

$$E(X) = \frac{2}{\pi} \sigma \quad (\text{S2-2})$$

The standard deviation  $\sigma$  can therefore be estimated as follows:

$$\sigma = \frac{\pi}{2} \bar{X} \quad (\text{S2-3})$$

Numerically, the standard deviation was found to be  $5.77 \times 10^{-3}$ .



**Figure S2-2:** Distribution of the difference between OD duplicates and fitting (plain line) to the half-normal distribution described by Equations S2-1a and S2-1b (with  $\sigma = 5.77 \times 10^{-3}$ ).

### 2.3. Calculation of the confidence interval on OD measurements

As OD measurements were made in duplicates, the OD values used in the formula of the viability (Equation 10 in the main manuscript) were the average ODs, expressed as:

$$OD_{av} = \frac{1}{N_r} \sum_i OD_i \quad (\text{S2-4})$$

where  $N_r$  is the number of replicates of a measurement (e.g. 2 for duplicates) and  $OD_i$  are the values of these replicates. As shown in the previous section, a sampled value of optical density  $OD_i$  can be assumed to follow a normal distribution centered on the ‘true’ value of the OD ( $OD_{true}$ ) and with a standard deviation  $\sigma$ .  $OD_{av}$  can therefore be assumed to follow a normal distribution centered around  $OD_{true}$  and characterized by a standard deviation  $\sigma/\sqrt{N_r}$ . The confidence interval on an OD measurement at 95% was therefore expressed as follows:

$$OD_{true} \in \left[ OD_{av} - 1.96 \frac{\sigma}{\sqrt{N_r}}; OD_{av} + 1.96 \frac{\sigma}{\sqrt{N_r}} \right] \quad (S2-5)$$

#### 2.4. Calculation of the confidence interval on the isotherm parameters

The error on OD measurement during the characterization of the isotherm caused uncertainty on the fitted values of the isotherm parameters. Monte-Carlo simulations were performed in order to quantify the level of accuracy on the parameters  $K$ ,  $q_m$ ,  $K_f$ , and  $n$ .

Monte-Carlo simulations consisted on iterating the fitting of the isotherm parameters a large number of times (10,000 in this case). At each iteration, the values of the optical densities for each data point were randomly sampled around the measured value and within the confidence interval given by Equation S2-5. The confidence interval for each isotherm parameter was then calculated from the standard deviation of the 10,000 values generated during the Monte-Carlo simulations.

#### 2.5. Calculation of the confidence interval on viability

According to Equation 10 in the main manuscript, there are two sources of uncertainty in the expression of the viability: the experimental error on the optical densities and the value of the parameter  $K'$ . The level of confidence on the optical densities was determined from Equation S2-5. The confidence interval on the parameter  $K'$  was taken equal to the confidence interval on  $K$  determined in section 2.4 multiplied by the constant  $\alpha$  (see the main Manuscript for details).

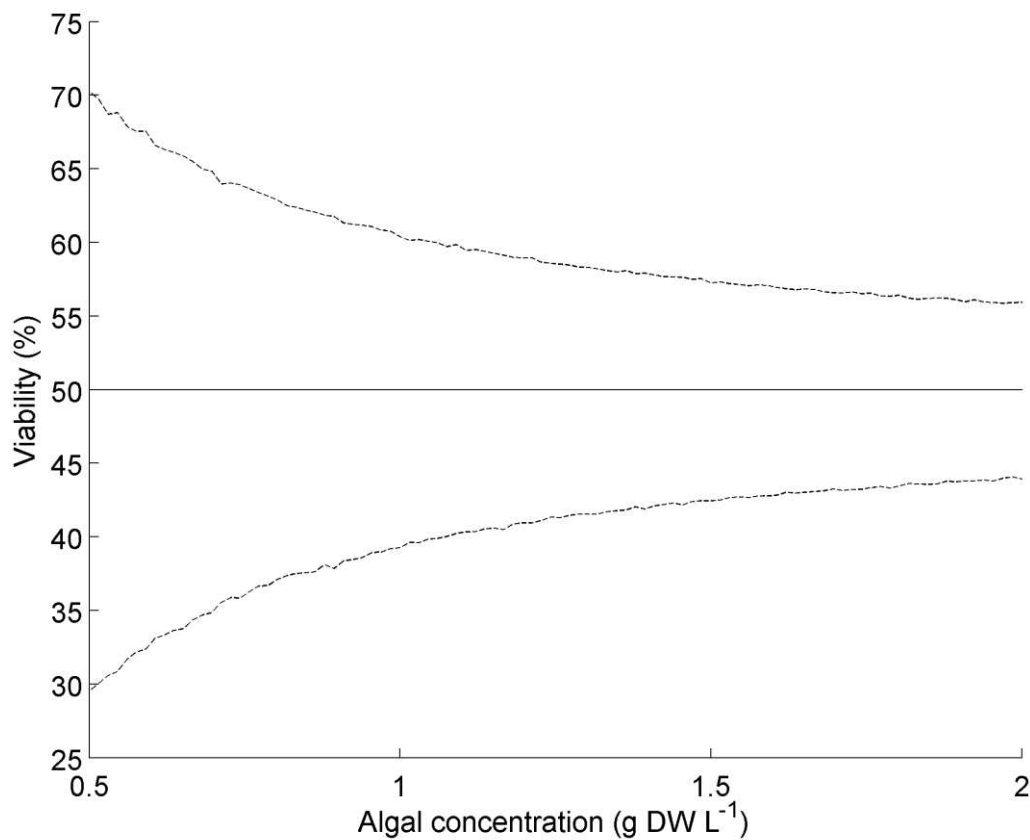
For each viability measurement, Monte-Carlo simulations consisted on repeating the calculation of the viability by using randomly generated values for the different ODs and the parameter  $K'$  in Equation 10. The level of confidence on the measured viability was then calculated from the standard deviation of the multiple resulting viability values (here 10,000 values) obtained at each iteration.

## *References*

Bland, J.M. 2005. The half-normal distribution method for measurement error: two case studies. Un-published talk available on <http://www-users.york.ac.uk/~mb55/talks/halfnor.pdf>).

### S3: Influence of the algal concentration on the uncertainty on viability

Figure S3-1 shows that the level of accuracy on the estimated viability on a half-dead algal sample significantly decreases when the algal concentration decreases.

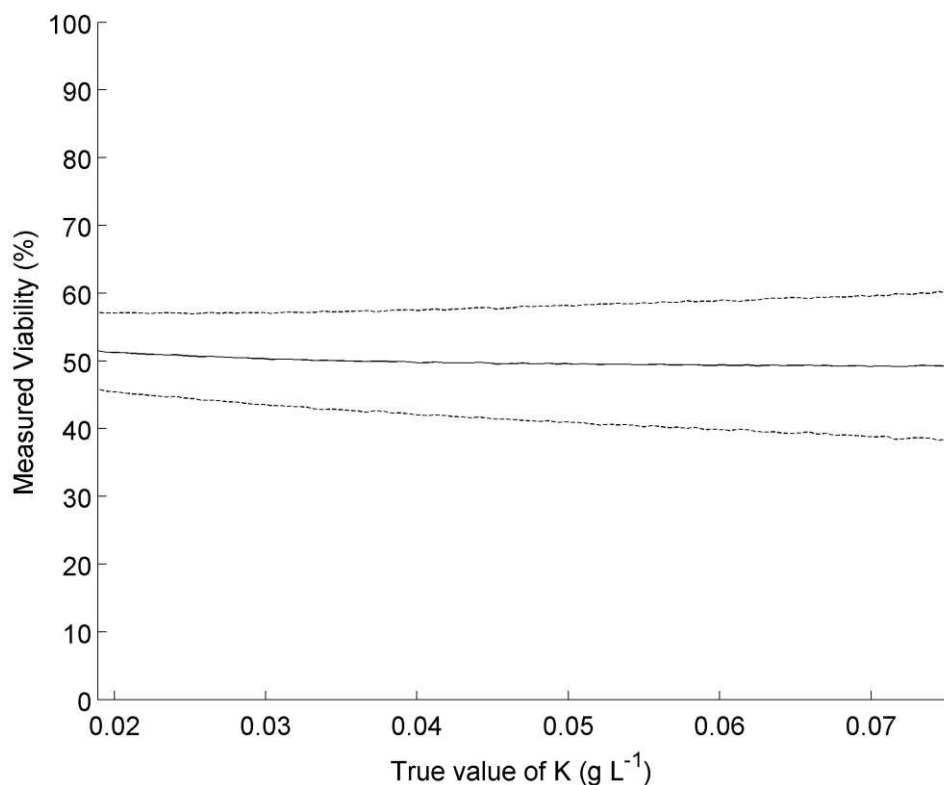


**Figure S3-1:** Measured viability (plain line) and associated confidence interval at 95% (dashed-line) of a hypothetical 50%-viable *C. vulgaris* sample for different algal concentrations (assuming that  $OD_S$  and  $OD_K$  are measured in duplicates and  $OD_B$  in triplicate). The algal concentrations represented on the x-axis is the concentration in the 0.6 mL added in the Sample and the Killed sample tubes. The error bars represent 95% confidence intervals estimated through Monte-Carlo simulations.

## S4: Impact of the uncertainty on $K$ on the viability results

As shown by Equation 10 in the main manuscript, estimating the viability of an algal sample requires determining the value of the half-saturation constant  $K$ . In this study, this parameter was assumed to be constant despite the potential changes of algal adsorption properties due to physiological changes (e.g. change of cell size, cell wall composition, etc.). This section investigates how the variability of this parameter can influence the results of the CAVA test.

In a hypothetical case, let us consider a 50% viable *C. vulgaris* sample. Figure S4-1 shows the viability of the sample estimated with a coefficient  $K$  of  $0.0378 \text{ g L}^{-1}$  as shown in Table 1 if the true value of  $K$  varies from 0.0189 (50%) to 0.0756 (200%). As shown by Figure S4-1, such a large variability in the value of  $K$  does not significantly affect the estimated viability. As a result,  $K$  can be assumed to be constant without significant loss of accuracy in viability.



**Figure S4-1:** Viability of a 50% viable *C. vulgaris* sample estimated with  $K = 0.0378 \text{ g L}^{-1}$  for different true  $K$  values (Hypothetical case:  $X = 1 \text{ g DW L}^{-1}$ ). Plain line: Estimated viability; dashed-line: confidence interval at 95%.