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► **To cite this version:**

Jérôme Cléach, Méline Soret, Thierry Grard, Philippe Lencel. Assessment of fish freshness based on fluorescence measurement of mitochondrial membrane potential. *Food Control*, 2020, 115, pp.107301. 10.1016/j.foodcont.2020.107301 . hal-03490842

HAL Id: hal-03490842

<https://hal.science/hal-03490842>

Submitted on 22 Aug 2022

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1 **Assessment of fish freshness based on fluorescence measurement of**
2 **mitochondrial membrane potential**

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12 **Highlights**

13 - Mitochondrial functions of European seabass fillets were disrupted from 96h storage (D4) at
14 +4°C

15 - Spectral properties of Rh123 and TMRM were correlated to mitochondrial health

16 - The evaluation of mitochondrial bioenergetics represents an early indicator of fish freshness

17 - A micro-volume fluorimeter could be considered for rapid evaluation of fish freshness

18

19 **Key words**

20 Mitochondrial potential; fish freshness; micro-volume fluorimetry

21 **Abstract**

22 Research and development of methods to assess fish freshness continues to be a major
23 challenge for the fishing industry. At the same time, consumers are now increasingly attentive
24 to food quality, including fish freshness and product history. Here, we propose a reliable,

25 rapid and easy-to-apply fluorimetric approach to assess fish freshness using a micro-volume
26 fluorimeter. Mitochondrial functions were assessed in European seabass (*Dicentrarchus*
27 *labrax*) fillets at different durations of storage at +4°C: from Day 0 to Day 8. We found that
28 mitochondrial respiration and mitochondrial membrane potential ($\Delta\Psi_m$) were significantly
29 disrupted after 4 days of storage at +4°C. The spectral properties (emission peak and
30 fluorescence intensity) of mitochondrial membrane potential probes rhodamine 123 (Rh123)
31 and tetramethylrhodamine methyl ester (TMRM) were strongly affected by the $\Delta\Psi_m$ integrity
32 of the fish fillets. We highlighted two categories of fish quality as a function of $\Delta\Psi_m$: Day 0 to
33 Day 3, $\Delta\Psi_m$ was preserved; and D4 to D7, $\Delta\Psi_m$ was disrupted. Thus, evaluation of $\Delta\Psi_m$
34 constitutes an early and reliable predictive indicator of fish freshness.

35 **1. Introduction**

36 Freshness is an important factor that contributes to fish quality (Olafsdottir, et al., 1997), and
37 its evaluation is still a major challenge in the fishing industry. Numerous methods and
38 technologies have been developed to evaluate fish freshness ((Mendes, 2018); (Wu, Pu, &
39 Sun, 2019) (Cheng, Sun, Han, & Zeng, 2014), (Hassoun & Karoui, 2015)). Current traditional
40 methods for assessing fish quality are based on physical, chemical, microbiological and
41 sensory parameters (Rehbein & Oehlenschlager, 2009). However, these methods present
42 numerous limits. They do not apply to all species, are time consuming, and require special
43 skills. Consequently, there is still a need in the food industry to develop rapid, reliable and
44 simple methods to assess fish freshness.

45 Understanding *post mortem* mechanisms in fish muscle cells is essential to identify new
46 markers, and to develop methods for the evaluation of fish freshness. In a previous study, we
47 assessed *post mortem* changes in mitochondrial functions and integrity in gilthead seabream
48 (*Sparus aurata*) fish muscle cells. We demonstrated that mitochondrial respiration and
49 mitochondrial membrane potential ($\Delta\Psi_m$) were significantly altered after 4 days of storage at

50 +4°C (Jérôme Cléach, et al., 2019). Our data showed, for the first time, that mitochondrial
51 activity constituted a putative reliable indicator to assess fish freshness at different early *post*
52 *mortem* time points.

53 In *post mortem* conditions, fish muscle is deprived of oxygen and nutrients, and is
54 consequently in an ischemic state. In this context, mitochondrial activity is disrupted. Many
55 methods are available to investigate mitochondrial functions and integrity, such as the
56 analysis of the mitochondrial respiratory chain by oxygraphy and the evaluation of
57 mitochondrial membrane potential ($\Delta\Psi_m$) by fluorimetry (Kuznetsov, et al., 2008).
58 Mitochondrial membrane potential ($\Delta\Psi_m$) and the proton gradient (ΔpH) constitute the
59 components of the proton motive force (Mitchell, 2011). $\Delta\Psi_m$ is essential for ADP
60 phosphorylation and ATP synthase activity. $\Delta\Psi_m$ is also involved in many less well studied
61 functions such as: homeostasis, mitochondrial function regulation, and transport of ions,
62 proteins, and nucleic acids (Zorova, et al., 2018). Therefore, monitoring oxygen consumption
63 by oxygraphy and evaluation of $\Delta\Psi_m$ are useful approaches to assess mitochondrial health.

64 The purpose of this study was to correlate *post mortem* mitochondrial bioenergetics with fish
65 freshness. This study focused on the assessment of $\Delta\Psi_m$ as a reliable method to determine fish
66 quality at early *post mortem* time points. We studied the $\Delta\Psi_m$ values of mitochondria isolated
67 from European seabass (*Dicentrarchus labrax*) skeletal muscle at different durations of
68 storage at +4°C to comply with the storage temperatures widely used in seafood processing
69 companies: from 6 hours after slaughtering (D0) to Day 7. First, in order to validate
70 mitochondrial extraction and assess mitochondrial respiration, we calculated the respiratory
71 control index (RCI) at different *post mortem* time points. Then, $\Delta\Psi_m$ was assessed using a
72 Nanodrop 3300 micro-volume fluorimeter (MVF). This device has many advantages; for
73 example, it is small, portable, easy-to-use and to maintain. These features make it ideal for
74 effective rapid evaluation of fish freshness in the food industry, without the need for special

75 technical skills. In a previous study, we demonstrated the strong capabilities of the MVF to
76 assess membrane potential with cationic fluorescent probes (J Cléach, et al., 2018). In this
77 study, we compared the action of two cationic fluorescent probes, traditionally used in the
78 study of mitochondrial potential: rhodamine 123 (Rh123) and tetramethylrhodamine methyl
79 ester (TMRM).

80 **2. Materials and methods**

81 2.1 Reagents

82 4-morpholinepropanesulfonic acid (MOPS), bovine serum albumin (BSA), carbonyl cyanide
83 3-chlorophenylhydrazone (CCCP), ethylene-bis(oxyethylenitrilo)tetraacetic acid (EGTA),
84 malate, proteinase type XXIV, rhodamine 123 (Rh123), succinate, tetramethylrhodamine
85 methyl ester perchlorate (TMRM), and Tris (hydroxymethyl)aminomethane (Trizma[®] base)
86 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium chloride (KCl) and
87 glutamate were acquired from Fisher Labosi (Paris, France). Magnesium chloride (MgCl₂),
88 potassium dihydrogen phosphate (KH₂PO₄) and sucrose were purchased from Acros Organics
89 (Morris, NJ, USA). Rh123, TMRM and CCCP were prepared in dimethyl sulfoxide (DMSO)
90 purchased from Thermo Scientific (San Diego, CA, USA).

91 2.2 Fish origin and storage

92 The European seabass (*Dicentrarchus labrax*) (300–450 g) used in this study were sourced
93 from Aquanord sea farm (Gravelines, France), as previously described (Jérôme Cléach, et al.,
94 2019). This farmed fish model was chosen in order to obtain accurate data on living
95 conditions, slaughtering, and storage, which can potentially influence the study of freshness.
96 Upon arrival at the laboratory, the fish were immediately filleted. The fillets were stored on
97 ice in a cold room (+4°C) for 8 days and used for experiments every 24 h over 8 days: Day 0,
98 Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, Day 7 and Day 8. The ice was renewed every day.

99 Plastic wrapping was used to avoid contact between the fillets and the ice or the accumulated
100 water.

101 2.3 Isolation of mitochondria from fish fillets

102 The method for mitochondria isolation was adapted from Pasdois, Parker, Griffiths, and
103 Halestrap (2011). It was also previously used for extraction of gilthead seabream (*Sparus*
104 *aurata*) skeletal muscle cell mitochondria (Jérôme Cléach, et al., 2019). All the steps in
105 mitochondrial isolation were performed in a cold room at +4 °C. Fish muscle was dissected
106 from the fillet (3 g) and finely diced with scissors. The fine pieces obtained (2–3 mm³) were
107 incubated at +4°C for 7 min under stirring in 20 mL of isolation buffer (180 mM KCl, 80 mM
108 sucrose, 5 mM MgCl₂, 10 mM Tris, 2 mM EGTA, pH 7.2 at +4°C), supplemented with 0.1
109 mg.mL⁻¹ of bacterial proteinase type XXIV. The resulting tissue suspension was poured into a
110 30 mL glass Potter homogenizer and homogenized for 3 min using a motorized Teflon pestle
111 at 300 rpm. The homogenate was centrifuged at 7,500 g for 10 min. The resulting pellet was
112 first washed and then resuspended in 20 mL isolation buffer containing 2 mg.mL⁻¹ of fatty
113 acid-free BSA, and homogenized for 3 min at 150 rpm. The homogenate was then centrifuged
114 at 700 g for 10 min. The supernatant was centrifuged at 1,500 g for 10 min. The resulting
115 supernatant was centrifuged again at 7,000 g for 10 min. The mitochondrial pellet obtained
116 was then suspended with a low volume (40 µL) of isolation buffer in order to obtain a
117 concentrated mitochondrial suspension. The protein concentration was determined using a
118 Bio-Rad protein assay kit, derived from the method developed by Bradford (1976), with BSA
119 as a standard. Mitochondria were kept on ice at a final concentration of 60–100 mg.mL⁻¹ for
120 not more than 4 h.

121 2.4 Respiratory control index (RCI)

122 Oxygraphy (Rank Brothers Digital Model 10, Cambridge, United Kingdom) was used to
123 monitor mitochondrial functions after isolation by measuring the respiratory control index

124 (RCI) at different days of storage at +4°C (Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6
125 and Day 7), as previously described (Jérôme Cléach, et al., 2019). First, 2.1 mL of respiration
126 buffer (KCl 125 mM, MOPS 20 mM, Tris 10 mM, EGTA 10 µM, KH₂PO₄ 2.5 mM, fatty
127 acid-free BSA 2 mg.mL⁻¹, pH 7.2) was added to the oxygraph chamber supplemented with
128 glutamate (5 mM), malate (2 mM), and succinate (5 mM). Then, mitochondria were added at
129 a final concentration of 0.2 mg.mL⁻¹. Oxygen consumption rates were assessed without and
130 with ADP (1 mM) (basal and state 3, respectively). Then, carboxyatractyloside (CAT) (5 µM)
131 was added to block the oxygen consumption linked to ATP synthesis. The experiment was
132 performed at +25°C. Saturating dithionite (Sigma-Aldrich) was added to the oxygraph
133 chamber to calibrate the device, and to achieve the zero oxygen calibration.

134 RCI was calculated using the following formula:

$$135 \quad RCI = \frac{ADP \text{ (state 3)}}{CAT}$$

136 Where state 3 is the respiration rate during maximum ATP synthesis, and CAT is the
137 respiration rate not linked to ATP synthesis.

138 2.5 Evaluation of mitochondrial membrane potential

139 A NanoDrop 3300 MVF (ThermoFisher Scientific, distributed by Ozyme, Saint-Cyr-l'École,
140 France) was used to monitor the fluorescence of Rh123 and TMRM in order to evaluate
141 changes in mitochondrial $\Delta\Psi_m$ of isolated mitochondria extracted at different storage
142 durations at +4°C (Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6 and Day 7). 1 mL of
143 respiration buffer (KCl 125 mM, MOPS 20 mM, Tris 10 mM, EGTA 10 µM, KH₂PO₄ 2.5
144 mM, fatty acid-free BSA 2 mg.mL⁻¹, pH 7.2) at +25°C was added to a 5 mL round-bottom
145 polypropylene tube, originally designed for flow cytometry analysis. Rh123 (50 nM final) or
146 TMRM (50 nM) were added to the respiratory buffer without respiratory substrates (as a
147 negative control) or with respiratory substrates: glutamate (5 mM), malate (2 mM), and
148 succinate (5 mM). Just prior to acquisition, isolated mitochondria were added at a

149 concentration of 0.2 mg.mL⁻¹. The decoupling agent CCCP (2 μM final) was then added to
150 the preparation to disrupt the ΔΨ_m, leading to Rh123 and TMRM output from the
151 mitochondria matrix.

152 Rh123 and TMRM were used in quenching mode. At high concentrations (50 – 100 nM),
153 cationic probes stack inside the mitochondrial matrix to form aggregates leading to the
154 quenching of the fluorescent emissions of the aggregated molecules (Perry, Norman, Barbieri,
155 Brown, & Gelbard, 2011).

156 2.6 Micro-volume fluorimeter settings

157 The MVF was originally designed to quantify nucleic acids and proteins. However, it is
158 possible to create and edit methods with the software to study a wide range of fluorophores. In
159 this way, we created a method to analyze the fluorescence of probes Rh123 and TMRM. The
160 excitation sources available include: UV LED (excitation maximum 365 nm), Blue LED
161 (excitation maximum 470 nm), and White LED (460-650 nm excitation). White LED was
162 selected to study Rh123 and TMRM fluorescence. The virtual emission filter interval (Δλ)
163 was set at ± 20 nm. 2 μL of sample were used to measure the fluorescence intensity. The
164 assay blank was carried out with the respiratory buffer. For each sample, 5 measures were
165 acquired, as recommended by the manufacturer. Importantly, this experimental approach did
166 not enable the user to measure ΔΨ_m, but gave a degree of alteration of mitochondrial
167 polarization. The measurement of fluorescence was reported in relative (non-absolute)
168 fluorescent units (RFU).

169 Rh123 and TMRM fluorescence intensities recorded at the two states of respiration (with or
170 without substrates) were normalized to the fluorescence recorded after CCCP addition,
171 according to the following formula:

$$172 \quad 100 - \left(\left(\frac{X - Y}{X} \right) * 100 \right)$$

173 Where: X = Rh123 or TMRM fluorescence intensity after CCCP addition

174 Y = Rh123 or TMRM fluorescence intensity after mitochondria addition with or
175 without substrates.

176 The calculated normalized fluorescence values using the above formula were based on the
177 maximum emission value. CCCP at 2 μ M completely depolarizes mitochondria by consuming
178 all the proton gradient established by the respiratory chain. As such, the fluorescence intensity
179 obtained after its addition corresponded only to the dissipation of $\Delta\Psi_m$, and made it possible
180 to take into account the non-specific binding of the dye.

181 2.7 Statistical analysis

182 The statistical analysis and graphs were generated with SPSS 17 software. Each experiment
183 was performed at least in triplicate. Data are expressed as mean \pm standard deviation.
184 Unpaired two-sample *t*-tests were used to express the significance of difference ($p < 0.05$)
185 between means, and Levene's test to determine the homogeneity of variance.

186 **3. Results**

187 *3.1 Changes in the respiratory control index*

188 First, mitochondrial functions in European seabass muscle cells were studied by assessing
189 mitochondrial respiration. To do this, the respiratory control index (RCI = ADP (state 3) /
190 CAT) was measured at different *post mortem* storage time points at +4°C (D0 to D8) (**Fig. 1**).
191 At D0, the RCI value was 7.3 ± 1.25 . At D2, RCI significantly decreased to reach 4.03 ± 1.15 .
192 From Day 4, the RCI value was near 1: D4 = 1.34 ± 0.33 and D8 = 1.08 ± 0.15 . In short, from
193 D0 to D4, RCI values gradually decreased to reach the minimum value at D4. From D4, *post*
194 *mortem* conditions led to mitochondrial respiration disruption.

195 *3.2 Changes in membrane potential of isolated mitochondria ($\Delta\Psi_m$) assessed with cationic* 196 *fluorescent probes*

197 The mitochondrial membrane potential of mitochondria isolated from European seabass
198 muscle cells was assessed at different storage time points: D0 to D7. The two cationic

199 fluorescent probes Rh123 and TMRM were used to evaluate $\Delta\Psi_m$. An MVF was required to
200 record dye fluorescence intensities. The fluorescence intensity of the cationic probes enabled
201 determination of the $\Delta\Psi_m$. When the mitochondrial functions and integrity were preserved,
202 the generation of $\Delta\Psi_m$ led to dye stacking inside the mitochondrial matrix. As a consequence,
203 the fluorescence properties of the cationic probes were affected: fluorescence intensity
204 decreased (quenching) and emission spectra shifted. In reverse, when mitochondria were
205 damaged, probe accumulation was weak and fluorescence quenching was low. Therefore, the
206 level of fluorescence of cationic probes was informative concerning mitochondrial health.

207 Mitochondria isolated from fish muscle at different *post mortem* time points were incubated
208 with Rh123 in the presence of respiratory substrates (glutamate, malate and succinate)
209 (**Fig. 2A: black lines**). As a negative control, the protonophore CCCP was added after
210 mitochondria staining to disrupt $\Delta\Psi_m$ (**Fig. 2A: red lines**). From D0 to D3, fluorescence
211 intensities were between 600 and 650 RFU. Then, from D4 to D5, they were higher and
212 reached 700 RFU. From D6 to D7, fluorescence was recorded at 800 RFU. Thus, from D0 to
213 D7, fluorescence gradually increased from 600 RFU to 800 RFU, highlighting *post mortem*
214 disruption of $\Delta\Psi_m$. From D0 to D7, the fluorescence intensity recorded after CCCP addition
215 was always around 900 RFU. This value constituted a reference corresponding to
216 mitochondria with disrupted $\Delta\Psi_m$. From D6 to D7, the values recorded without CCCP (black
217 curve) were close to those recorded after CCCP addition (red line), showing the strong
218 disruption of $\Delta\Psi_m$. As a negative control, we also stained isolated mitochondria without
219 respiratory substrates, to confirm that the fluorescence quenching was specifically due to the
220 energization of mitochondria (data not shown). A blue line has been drawn at 600 RFU to
221 visualize the significant increasing change in fluorescence intensity from D0-D3 to D4-D7.
222 These data show $\Delta\Psi_m$ disruption in the European seabass fish muscle mitochondria after 96
223 hours of storage at +4°C

224 In order to confirm our results with the Rh123 probe, we used a second cationic fluorescent
225 probe sensitive to $\Delta\Psi_m$. Mitochondria isolated from European seabass muscle cells were
226 stained with TMRM, following the same protocol used with the Rh123 probe. A typical
227 fluorescence profile of TMRM after isolated mitochondria labelling was found at different
228 *post mortem* time points (**Fig. 2B: black lines**). As a negative control, the protonophore
229 CCCP was added after mitochondria staining to disrupt $\Delta\Psi_m$ (**Fig. 2B: red lines**). From D0 to
230 D3, fluorescence intensities were between 700 and 750 RFU. Then, from D4 to D5,
231 fluorescence was between 1,000 and 1,100 RFU. From D6 to D7, fluorescence reached 1,200
232 RFU. As such, from D0 to D7, fluorescence gradually increased from 700 RFU to 1,200 RFU,
233 highlighting *post mortem* disruption of $\Delta\Psi_m$. From D0 to D7, the fluorescence intensities
234 recorded after CCCP addition were between 1,200 and 1,400 RFU. This range of values
235 constituted a reference corresponding to mitochondria with disrupted $\Delta\Psi_m$. From D6 to D7,
236 the values recorded without CCCP (black curve) were close to those recorded after CCCP
237 addition (red line), showing the strong disruption of $\Delta\Psi_m$. As a negative control, we also
238 stained isolated mitochondria without respiratory substrates to confirm that the fluorescence
239 quenching was specifically due to the energization of mitochondria (data not shown). A blue
240 line has been drawn at 700 RFU to visualize the significant decreasing change in the
241 quenching fluorescence from D0-D3 to D4-D7. As for Rh123 staining, the data recorded with
242 the TMRM probe demonstrated $\Delta\Psi_m$ disruption after 96 hours of storage at +4°C. Therefore,
243 the results obtained with the TMRM probe were correlated with those recorded with the
244 Rh123 probe.

245 *3.3 Statistical analysis of changes in the fluorescence of cationic probes Rh123 and TMRM at* 246 *different post mortem time points*

247 The experiments illustrated in Figures 2A and 2B were performed at least three times in order
248 to acquire a statistical overall view of changes in $\Delta\Psi_m$ at different durations of fish fillet

249 storage (**Fig. 3**). For both probes, fluorescence intensity recorded for each day was normalized
250 to CCCP. Importantly, the cationic probes Rh123 and TMRM are sensitive to the $\Delta\Psi_m$.
251 However, they can also interact non-specifically with other organelles such as the
252 endoplasmic reticulum, lysosomes, and other cell fragments (Cottet-Rousselle, Ronot,
253 Leverage, & Mayol, 2011). This addition of CCCP allowed us to record a fluorescence
254 intensity in mitochondria with a disrupted mitochondrial membrane. This approach of
255 rationalization with CCCP therefore enabled us to take into account the non-specific binding
256 of the dyes.

257 **Fig. 3A** illustrates changes in Rh123 fluorescence normalized to CCCP at different *post*
258 *mortem* time points. From D0 to D3, normalized Rh123 fluorescence was between 65 and 75
259 %. From D4 to D7, fluorescence was around 85 %. Two zones delimited by a dotted line at 75
260 % of fluorescence were identified. This dotted line highlighted a significant change in $\Delta\Psi_m$
261 between the two periods: “D0-D3” and “D4-D7”.

262 Changes in TMRM fluorescence were also normalized with CCCP at the same *post mortem*
263 time points as for the Rh123 probe (**Fig. 3B**). From D0 to D3, normalized TMRM
264 fluorescence was between 60 and 70 %. From D4 to D7, fluorescence was between 80 and 95
265 %. Like for the statistical data obtained with the Rh123 probe, a dotted line traced at 75 %
266 delimited two zones, and highlighted a significant change in $\Delta\Psi_m$ between the two periods:
267 “D0-D3” and “D4-D7”.

268 *3.4 Evolution of the Rh123 and TMRM fluorescence emission peak wavelengths at different* 269 *post mortem time points*

270 Cationic probe characteristics such as the emission peak wavelength depend on mitochondrial
271 functions and integrity ((Emaus, Grunwald, & Lemasters, 1986); (Scaduto Jr & Grotyohann,
272 1999)). For each condition and both probes, emission peak wavelengths were studied with the
273 MVF (**Fig. 4**).

274 For a given typical experiment with energized mitochondria stained with Rh123, the
275 fluorescence emission peaks were recorded for each day of storage (**Fig. 4A**). From D0 to D2,
276 the emission peak wavelength (EPW) was 528 nm. From D3 to D4, the EPW was 526 nm.
277 Then, from D5 to D7, the EPW was 524 nm. Consequently, from D0 to D7, the emission peak
278 shifted from 528 nm to 524 nm. As a control, emission fluorescence was measured after
279 addition of CCCP (524 nm).

280 The emission peak was also recorded at different days of storage after TMRM staining
281 (**Fig. 4B**). From D0 to D3, the EPW was 579 nm. From D4 to D5, the EPW was 577 nm.
282 Then, from D6 to D7, the EPW was 576 nm. Consequently, for a given experiment with
283 energized mitochondria, the TMRM emission profile changed, with a shift from 579 nm to
284 576 nm from D0 to D7. As a negative control, when mitochondria were decoupled after
285 CCCP addition, the EPW was 576 nm.

286 For both probes, the EPWs were measured for each duration of storage and compared by
287 statistical analysis (**Fig. 4C, 4D**). For Rh123 staining (**Fig. 4C**), when mitochondria were
288 energized, the EPW was higher than 526 nm (527-529 nm) from D0 to D3, and less than 526
289 nm after 4 days of storage (D4 to D7). A dotted red line on the graph delimited and
290 highlighted the significant difference between the two zones. When energized mitochondria
291 were stained with CCCP, emission fluorescence peaks were measured between 524 and 525
292 nm. As a control, peak emission fluorescence was also recorded between 524 and 525 nm in
293 the absence of substrates (data not shown). These data demonstrated the specific action of
294 energized mitochondria on the characteristic emission peak. As a result, the phenomena of
295 red-shift was only visible with energized mitochondria with respiratory substrates. The same
296 observations were made for TMRM staining (**Fig. 4D**), except that the wavelength
297 significantly discriminating D0-D3 from D4-D7 was located at approximately 577.5 nm (**red**
298 **dotted line: Fig. 4D**).

299 **4. Discussion**

300 The development of methods to evaluate fish freshness and thereby its quality is currently still
301 a challenge and an issue in the fishing industry. In a previous study (Jérôme Cléach, et al.,
302 2019), we demonstrated that mitochondrial functions and integrity constitute reliable and
303 early indicators to evaluate gilthead seabream freshness. In the present study, we focused our
304 experiments on the evaluation of mitochondrial membrane potential ($\Delta\Psi_m$). To this end, we
305 followed a new approach using an MVF to evaluate $\Delta\Psi_m$ with cationic fluorescent probes. In
306 order to study the universality of this method, we performed our experiments on another
307 farmed species: the European seabass (*Dicentrarchus labrax*). Our results provided
308 knowledge on *post mortem* mitochondrial bioenergetics in skeletal muscle cells.

309 *4.1 Post mortem respiration of mitochondria isolated from fish fillets*

310 As a first approach, we studied mitochondrial functions of European seabass fillet muscle
311 cells using oxygraphy. The RCI value was calculated to evaluate mitochondrial bioenergetics.
312 Under given conditions, high RCI indicated good mitochondrial functions, and on the
313 contrary low RCI usually reflected mitochondrial dysfunction (Brand & Nicholls, 2011). RCI
314 was therefore a good indicator of mitochondrial health. RCI values were calculated at
315 different *post mortem* durations of storage: D0, D2, D3, D4 and D8. From D0 to D4, RCI
316 values gradually decreased. From D4 to D8, the minimum value of RCI was reached,
317 demonstrating mitochondrial decoupling. In our previous study (Jérôme Cléach, et al., 2019),
318 the RCI was evaluated using mitochondria isolated from farmed gilthead seabream (*Sparus*
319 *aurata*) at different durations of storage at +4°C. Comparing the results obtained for both
320 species, we observed that RCI values were higher in the European seabass model than in the
321 gilthead seabream model during the initial *post mortem* days. This can be explained by the
322 fact that RCI values depend on and vary as a function of species and tissues (Hulbert, Turner,
323 Hinde, Else, & Guderley, 2006). However, for both species, day 4 constituted the critical

324 storage day where mitochondrial functions were significantly disrupted (respiration and
325 $\Delta\Psi_m$).

326 On the one hand, RCI measurement allowed us to validate the high quality of our
327 mitochondrial extraction protocol. On the other, it made it possible to establish a correlation
328 between mitochondrial oxygen consumption and $\Delta\Psi_m$ at different *post mortem* time points.

329 4.2 Post mortem membrane potential of mitochondria isolated from fish fillets

330 As a second approach, $\Delta\Psi_m$ was assessed at different days *post mortem* using a fluorimetric
331 method, like for oxygraphy. From D0 to D3, isolated mitochondria conserved their $\Delta\Psi_m$ in
332 the presence of respiratory substrates. After 96 h of storage (D4), $\Delta\Psi_m$ was significantly
333 disrupted. As a result, there was correlation between changes in mitochondrial respiration and
334 $\Delta\Psi_m$ in fish muscle cells in *post mortem* conditions. In our previous study, with the gilthead
335 seabream model, we also demonstrated disruption of $\Delta\Psi_m$ at D4. Thus, for both species,
336 mitochondrial bioenergetics were significantly altered at the same *post mortem* time points.
337 Therefore, D4 represented a “critical” storage time where mitochondrial respiration and $\Delta\Psi_m$
338 were significantly and strongly disrupted in both species. This similarity of results can be
339 explained by the fact that these two species present numerous common points. Importantly,
340 they have the same lifestyle. Moreover, the tested fish originated from the same aquaculture
341 farm and consequently had the same nutrition, the same living environment, and were
342 slaughtered by the same method. All these parameters could probably have a direct impact on
343 *post mortem* changes in mitochondrial functions and integrity.

344 4.3 The post mortem mitochondrial survival strategy

345 RCI decreased significantly during the first 3 days, with maintenance of the mitochondrial
346 membrane potential over the same period. On the basis of these results, from D0 to D3,
347 mitochondria isolated from European seabass fillet muscle cells retained their membrane
348 potential and the integrity of the respiratory chain. Over the first 3 days, the RCI decreased,

349 illustrating that ADP could no longer accelerate respiratory chains. These results correlate
350 with those previously obtained on gilthead sea bream (Jérôme Cléach, et al., 2019). From D4
351 to D8, the RCI decreased significantly and was accompanied by a reduction in membrane
352 potential to reach its minimum value.

353 The maintenance of $\Delta\Psi_m$ in *post mortem* conditions (8.5 hours and 24 hours, respectively)
354 was previously reported by Barksdale, et al. (2010) in other species. In *post mortem*
355 conditions, muscle cells are deprived of oxygen and nutrients. Similarly, it was reported that
356 25% of oxygen was still present in beef *longissimus thoracis et lumborum* a few minutes after
357 slaughtering. After 48 hours of storage at +4°C, 10% of the oxygen was still present in beef
358 muscle (England, et al., 2018). As mentioned in this study, even a low amount of oxygen and
359 a low number of viable mitochondria can participate in *post mortem* metabolism. We can
360 suppose that a certain percentage of oxygen is also present in fish fillets after slaughtering,
361 and that this allows for survival of mitochondria. Previous studies have clearly shown the key
362 role of mitochondria in *post mortem* metabolism ((St-Pierre, Brand, & Boutilier, 2000),
363 (Nicholas J. Hudson, 2012), (England, et al., 2018)). Therefore, mitochondria are selfish and
364 prioritize their own survival by consuming ATP reserves in order to maintain their
365 homeostasis and functionality ((St-Pierre, et al., 2000), (Lehmann, Segal, Muradian, &
366 Fraifeld, 2008), (Nicholas J. Hudson, 2012)). This process of mitochondrial survival in anoxia
367 is called “mitochondrial treason” (N. J. Hudson, et al., 2017).

368 *4.4 Spectral properties of Rh123 and TMRM as indicators of fish freshness*

369 The originality of this study was based on a new approach to assess mitochondria with an
370 MVF. This device made it possible to acquire fluorescence emission spectra. We observed
371 that Rh123 and TMRM fluorescence emissions were strongly impacted as a function of *post*
372 *mortem* time points. Two characteristics of Rh123 and TMRM are to be considered to
373 evaluate fish freshness: the level of quenching fluorescence and the emission peak

374 wavelength. From D0 to D3, for both probes, the levels of quenching were higher at the D0-
375 D3 interval than at the D4-D7 interval. Moreover, the emission fluorescence peak shifts were
376 only observable at D0-D3, also for both probes. Therefore, mitochondrial probe fluorescence
377 and emission peaks were linked to the “energized state” of mitochondria. Emaus, et al.,
378 (1986) were the first to highlight the fluorescence properties of Rh123 using uncoupled and
379 coupled rat isolated mitochondria. They considered spectral red shifts and fluorescence
380 quenching to be a consequence of electrophoretic dye uptake, followed by high capacity
381 binding in the mitochondrial matrix in coupled mitochondria, but not in uncoupled
382 mitochondria. The same observation was also reported in another study with TMRM
383 ((Scaduto Jr & Grotyohann, 1999)). These authors showed that TMRM excitation and
384 emission spectra change, as shown by quenching and peak emission shift, after incubations of
385 heart rat mitochondria with dye once energized with glutamate, malate and succinate. As
386 proposed by Diop, et al. (2016), we can also classify in this study a fish fillet in the D0-D3
387 zone or in the D4-D7 zone, on the basis of high quenching and shift of the fluorescence peak
388 or high quenching and no shift of the fluorescence peak, respectively. In this way, we
389 highlighted new markers to evaluate fish freshness. In the future, it may be useful to
390 investigate whether the fluorescence quenching and the emission peak wavelength shifts
391 occur in the case of labeling of cells or tissues, as for isolated mitochondria.

392 **5. Conclusions**

393 In this study, the respiratory activity and $\Delta\Psi_m$ of mitochondria isolated from European
394 seabass fillet were assessed at different *post mortem* time points. Mitochondria were stained
395 with the cationic probes Rh123 and TMRM, and fluorescence was recorded with an MVF.
396 We found dysfunction of mitochondrial bioenergetics after 96 hours of storage at +4°C (D4).
397 Moreover, the spectral properties of the probes, such as emission peak wavelength and
398 fluorescence intensity, were significantly affected from D4. This study therefore confirmed

399 that evaluation of mitochondrial bioenergetics represents a reliable and early indicator of fish
400 freshness among the various existing indicators. It would be interesting in the future to
401 establish a “specific freshness profile” for different fish species, in particular for fish with
402 high commercial value such as Atlantic salmon (*Salmo salar*) or rainbow trout
403 (*Oncorhynchus mykiss*).

404 **Funding**

405 This study was funded by the French government, Ifremer and the Hauts-de-France region in
406 the framework of the CPER 2014-2020 MARCO project.

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478

479 **Figures caption**

480 **Fig. 1:** Changes in the respiratory control index (RCI) of mitochondria isolated from
481 European seabass fillet muscle cells at different *post mortem* storage times at +4°C
482 Letters denote values that are significantly different at different storage times. The *t*-test was
483 performed using the SPSS Statistics 17 system. The different letters (a, b, c, d) above each bar
484 represent significant differences; ($p < 0.05$; $n=3$).

485

486 **Fig. 2:** Changes in $\Delta\Psi_m$ of European seabass fillet muscle cell-isolated mitochondria assessed
487 with the Rh123 (A) or TMRM (B) probes

488 A: Rh123 probe: relative fluorescence units were measured at different time points of *post*
489 *mortem* storage at +4°C (from Day 0 to Day 7). $\Delta\Psi_m$ was evaluated in the presence of
490 substrates without (dark lines) or with (red lines) CCCP. The blue line highlights the
491 significant change in membrane potential between the periods D0 to D3 and D4 to D7

492 B: TMRM probe: relative fluorescence units were measured at different time points of *post*
493 *mortem* storage at +4°C (from Day 0 to Day 7). $\Delta\Psi_m$ was evaluated in the presence of
494 substrates without (dark lines) or with (red lines) CCCP. The blue line highlights the
495 significant change in membrane potential between the periods D0 to D3 and D4 to D7.

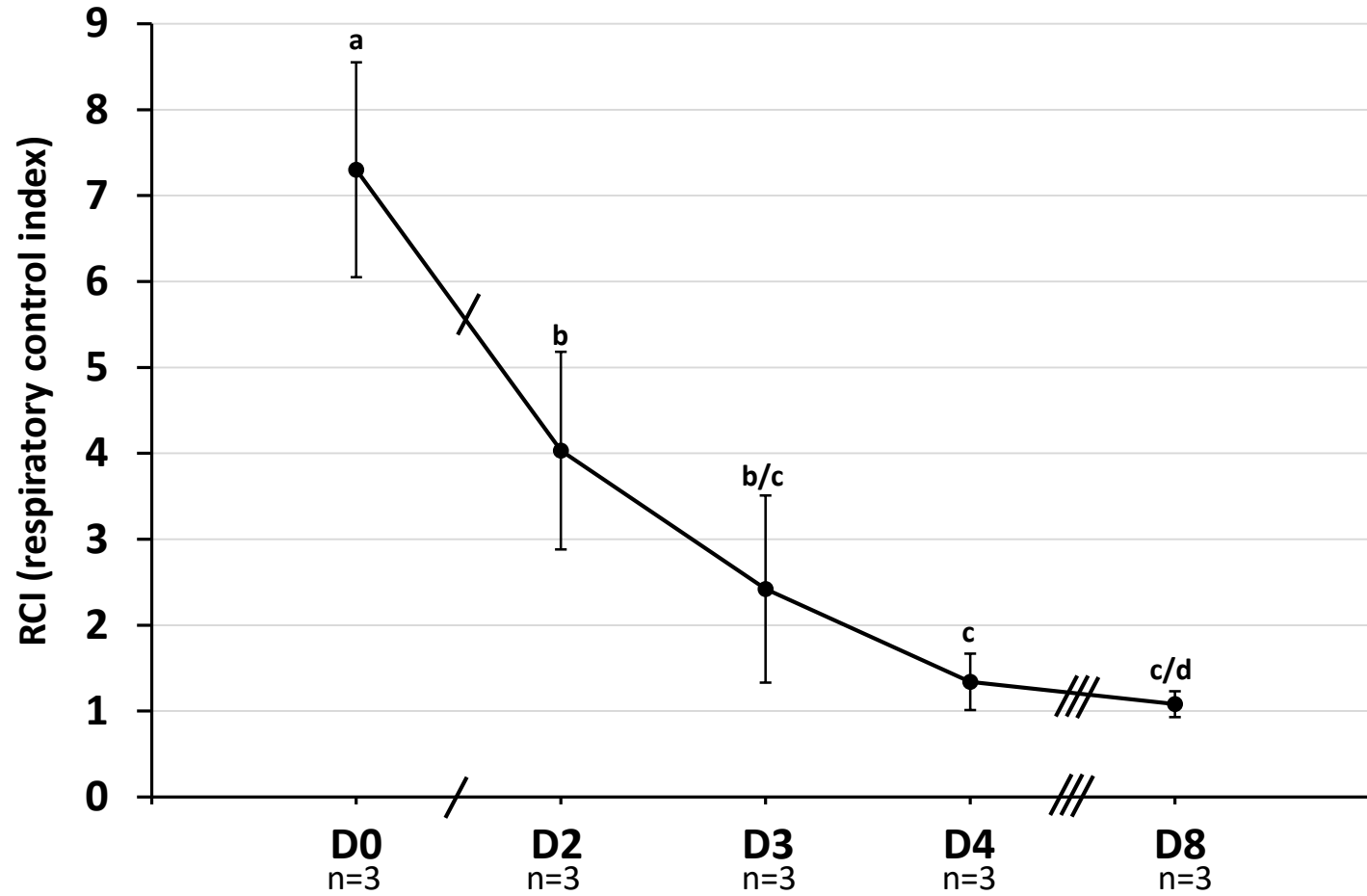
496

497 **Fig. 3:** Summary graphs of Rh123 (A) or TMRM (B) fluorescence levels normalized to CCCP
498 at different storage times

499 The different letters (a, b, c) represent significant differences; ($p < 0.05$; $n=3-6$). The dotted
500 line highlights the significant change in membrane potential between the periods D0 to D3
501 and D4 to D7.

502

503 **Fig. 4:** Changes in the Rh123 (A) and TMRM (B) fluorescence emission peak wavelengths as
504 a function of different storage times of seabass fillets and their statistical representations (C
505 and D)
506 Figures A and B are representative of a typical experiment.
507 The different letters (a, b, c, d) above each bar (C and D) represent significant differences; (p
508 < 0.05 ; $n=3$).
509 The blue vertical lines indicate the wavelengths 528 nm (A) or 579 nm (B), corresponding to
510 the fluorescence peak after staining of mitochondria isolated from fish fillet muscle cells
511 between D0 and D3.
512 The grey vertical lines indicate the wavelengths 524 nm (A) or 576 nm (B), corresponding to
513 the fluorescence peak after staining of mitochondria isolated from fish fillet muscle cells
514 between D4 and D7.
515 The red vertical dotted lines (A and B) highlight the limit between two mitochondrial
516 functional states.
517 The blue curves (C and D) illustrate changes in the fluorescence emission peak wavelengths
518 in the presence of stained mitochondria from D0 to D7.
519 The grey curves (C and D) illustrate changes in the fluorescence emission peak wavelengths
520 in the presence of stained mitochondria and CCCP from D0 to D7.
521 The red horizontal dotted lines (C and D) highlight the limit between two mitochondrial
522 functional states.

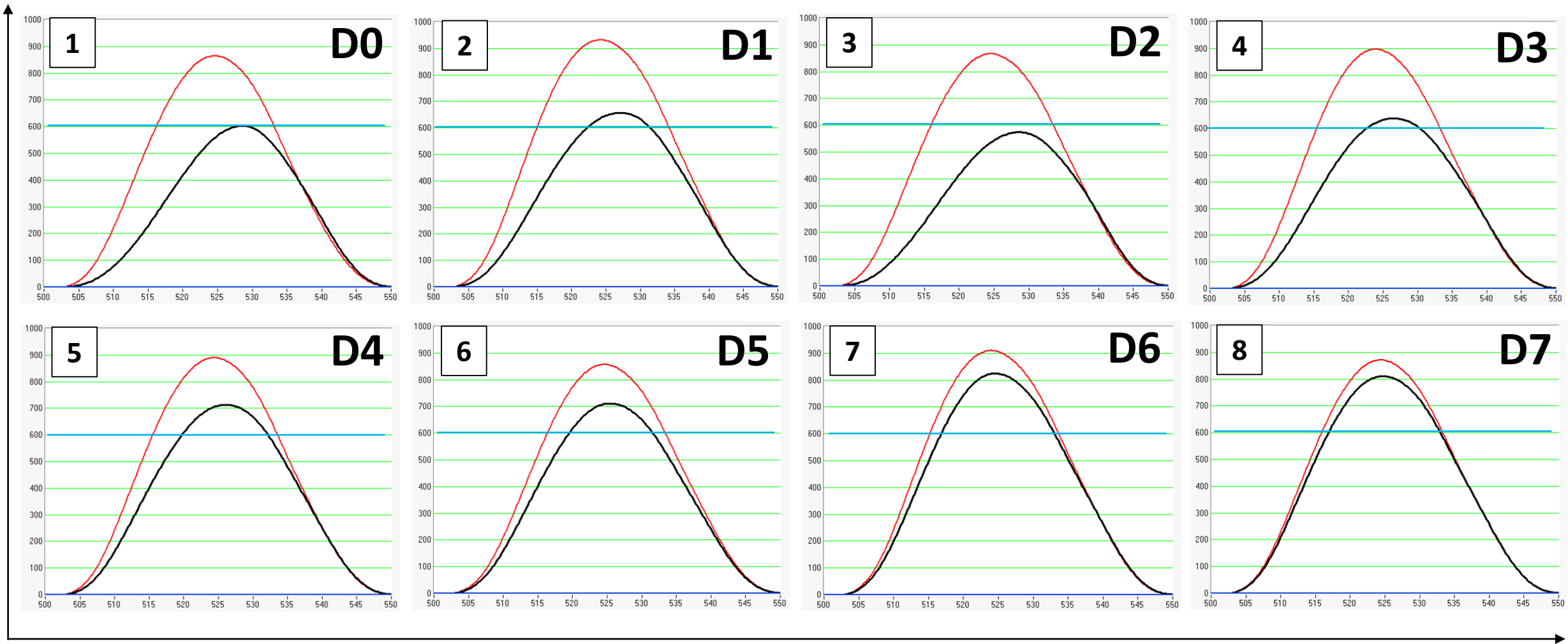


<i>Day of storage</i>					
	D0	D2	D3	D4	D8
<i>RCI value</i>	7.3 ± 1.25	4.03 ± 1.15	2.42 ± 1.09	1.34 ± 0.33	1.08 ± 0.15

Rh123

A

Relative fluorescence units (RFU)



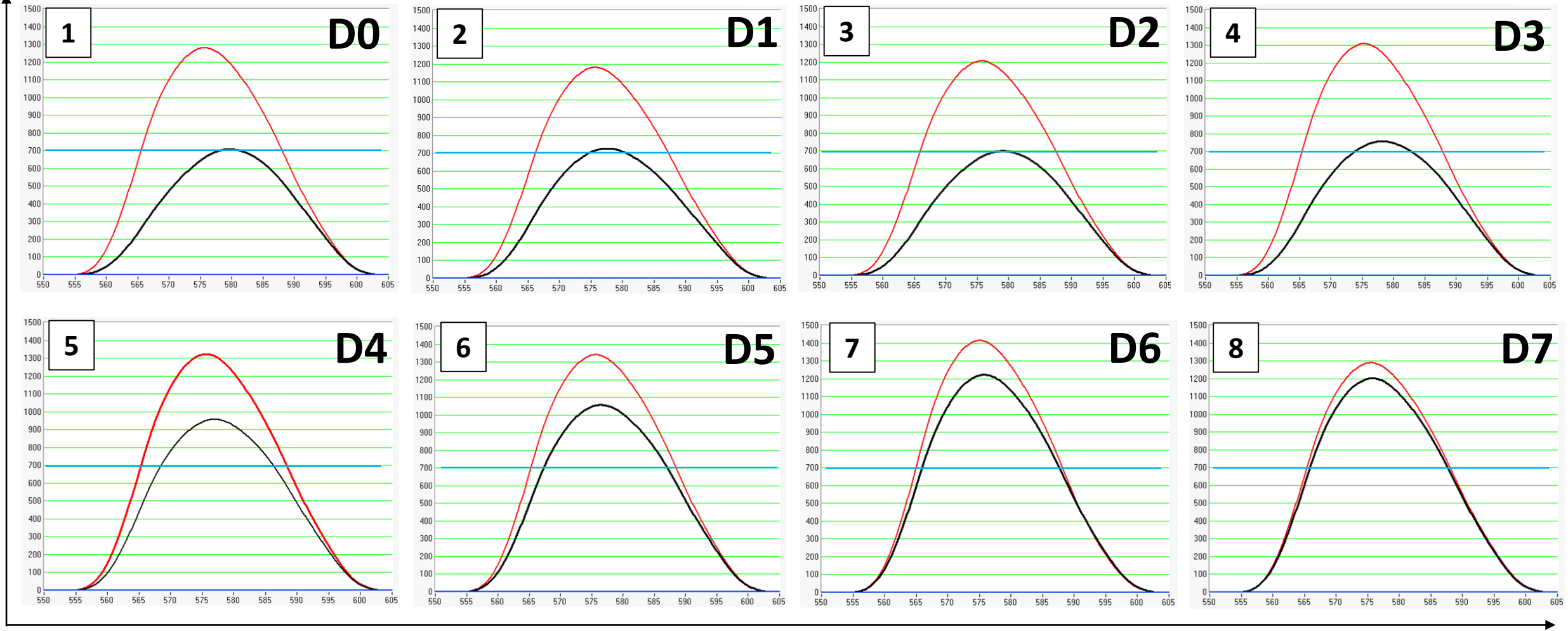
Wavelengths (nm)

— Substrates without CCCP — Substrates with CCCP

TMRM

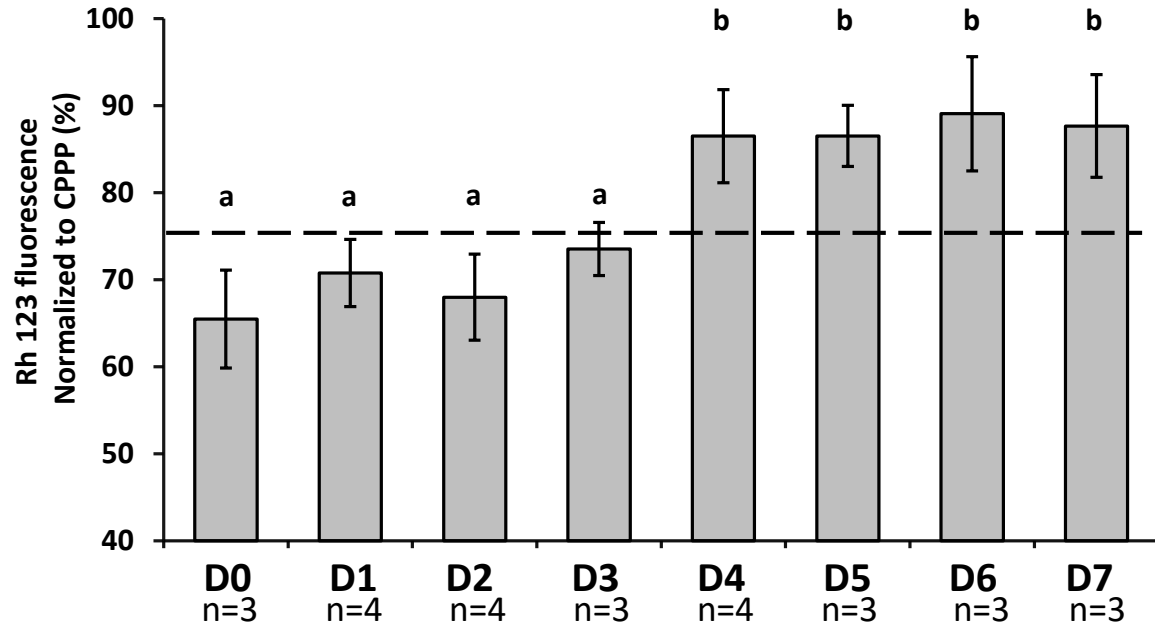
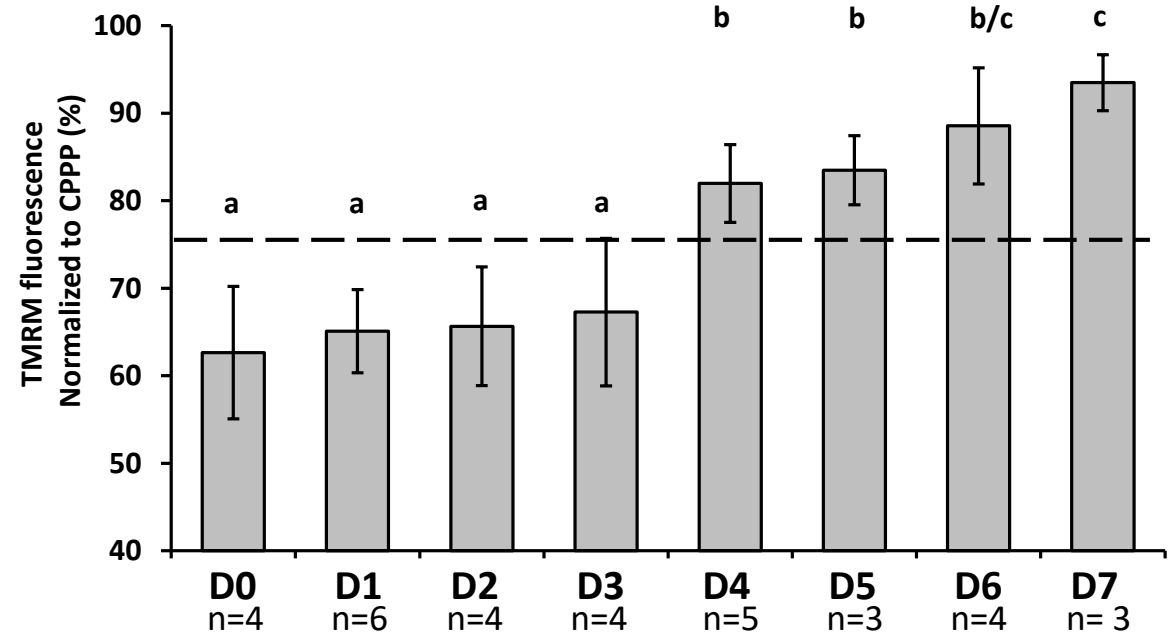
B

Relative fluorescence units (RFU)



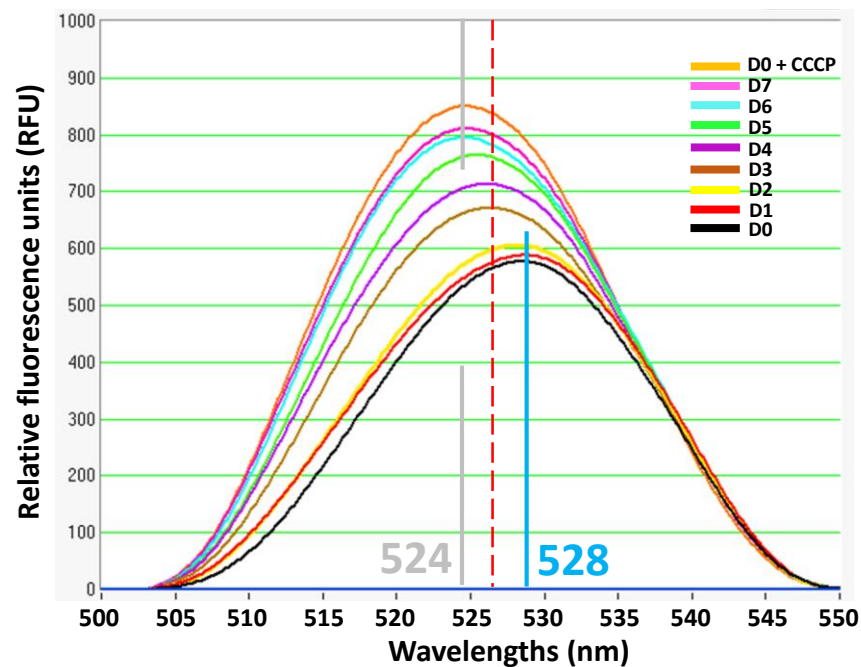
Wavelengths (nm)

— Substrates without CCCP — Substrates with CCCP

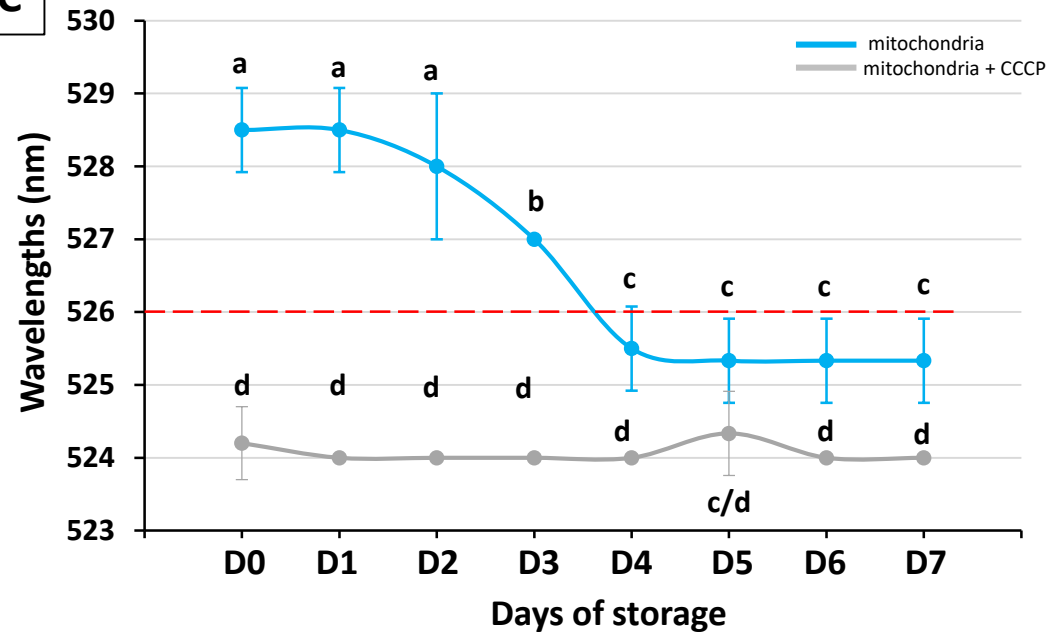
A**Rh123****B****TMRM**

Rh123

A

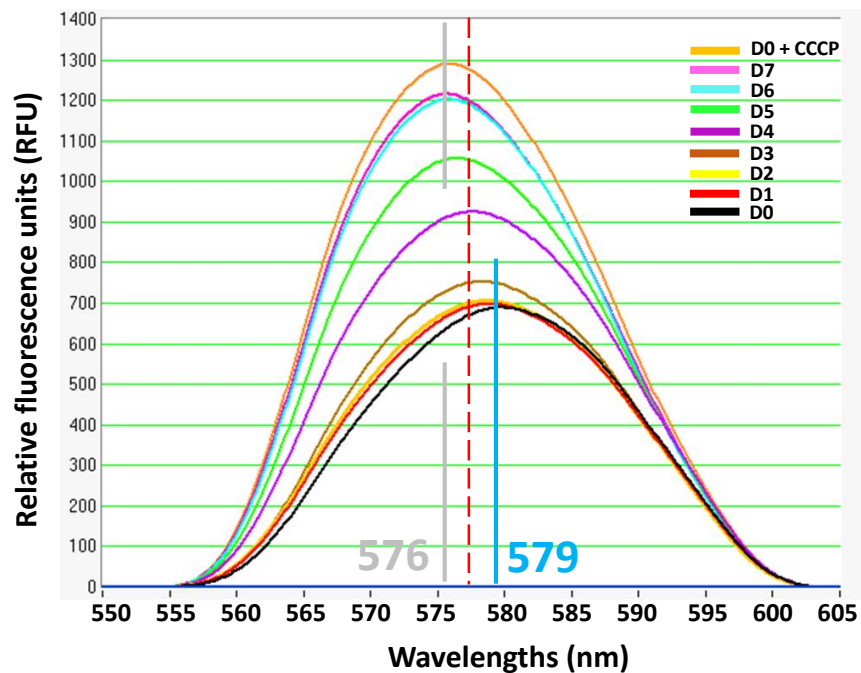


C



TMRM

B



D

