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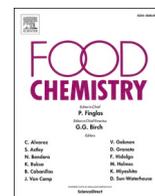
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Measurement of fish freshness: Flow cytometry analysis of isolated muscle mitochondria

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ABSTRACT

Mitochondria are real sensors of the physiological status of tissues. After the death of an animal, they maintain physiological activity for several days. This activity is highly dependent on the availability of nutrients in the tissue. In this study, flow cytometry was used to measure the membrane potential of mitochondria isolated from European seabass (*Dicentrarchus labrax*) red muscle stored in ice for seven days in order to characterize fish freshness. Two probes, TMRM and Rhodamine 123, were used to measure mitochondrial potential. During the first few days (D0 to D3), isolated mitochondria maintained high potential, and then lost their potential (from D3 to D5), but were always re-polarizable after addition of substrates (glutamate, malate and succinate). From D7, the mitochondria were more strongly depolarized and were difficult to repolarize by the substrates. Using flow cytometry, we demonstrated that mitochondria were an excellent marker to confirm seabass freshness.

1. Introduction

The impact of food quality on human health is critical. Government agencies are responsible for ensuring food safety, paying particular attention to chemical and microbiological risks (Kher et al., 2013). The accumulation of knowledge on this subject has made it possible to change the way consumers and manufacturers look at food or food matrices. Consumers have many different ways of perceiving products, but their perception is increasingly linked to health benefits (Grunert, Bredahl, & Brunso, 2004; Kamihiro, Stergiadis, Leifert, Eyre, & Butler, 2015). In parallel, increasing interaction between consumers and industry is leading to changes in practices in the agri-food sector and more particularly in the aquatic products industry (Wu, Pu, & Sun, 2019).

The quality of aquatic products is still based exclusively on chemical and microbiological criteria. Even though the number of methods for measuring seafood quality is increasing, the methods that are used to measure fish freshness are still based on the total volatile base nitrogen (TVB-N) level (Parlapani, Mallouchos, Haroutounian, & Bozariis,

2014) or on the determination of biogenic amines (Pinto, Nieto, Zón, Fernández, & De Araujo, 2016). These are reference methods which, although used routinely, yield variable results depending on the species.

Mitochondria seem to play an important role in changes in the quality of food products, in particular by activating proteases and by producing numerous free radicals which are involved in how products change *post mortem* (England et al., 2018; Sierra & Oliván, 2013). These oxidation reactions are involved in the modification of the nutritional quality and indirectly impact human health (Huang & Ahn, 2019). Various studies have reported *post mortem* morphological mitochondrial changes and some have shown that mitochondrial activity was still present *post mortem* in storage conditions of + 4 °C in bovine, pork, poultry or fish models (Chauhan & England, 2018; Tang et al., 2005). Mitochondrial activity was also used to measure seafood alteration by monitoring the consumption or production of metabolites (Chang, Wu, Yeh, & Liao, 2020; Hassoun & Karoui, 2017).

As part of a previous study examining measurements of early markers of freshness, we showed that measuring fish freshness could be

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associated with measuring mitochondrial activity (Cléach et al., 2019). The structural and physiological changes in mitochondria in fish fillets stored at 4 °C seemed to show that mitochondria are a good indicator of freshness and spoilage, as well as the good physiological condition of the cells or tissues.

In another previous study, we showed that mitochondria isolated from gilthead seabream (*Sparus aurata*) fillets maintained normal respiratory activity for 3 days. Uncoupling was observed on the fourth day with a gradual loss of mitochondrial membrane potential (Cléach, Soret, Grard, & Lencel, 2020).

This study completed the triptych of approaches developed in the laboratory concerning the determination of fish freshness by studying variations in *post mortem* mitochondrial potential according to the following three approaches: oxygraphy (Cléach et al., 2019); fluorescence (Cléach et al., 2020) and flow cytometry (current article).

In this study, we extended this observation to another fish species: seabass (*Dicentrarchus labrax*). The approach was based on a flow cytometric analysis of mitochondria isolated from seabass fillets stored at 4 °C. Flow cytometry allowed us to target both an organelle (mitochondria) and a marker of choice (mitochondrial potential). For the first time, we demonstrate that flow cytometric analysis of mitochondrial potential is a determinant of the level of spoilage of fish fillets.

Flow cytometry is used specifically in the food field to evaluate microorganisms survival, metabolic activities, and stress, both in processing and in finished products ((Malacrinò, Zapparoli, Torriani, & Dellaglio, 2001)). A large number of fluorescent probes are available to assess membrane permeability, enzymatic activities, bacterial potential, and various cellular constituents (Sträuber & Müller, 2010).

The aim of this study was to monitor the status of seabass fillets by flow cytometry analysis of mitochondria isolated from these fillets. This method has already been widely used to assess the impact of physical or chemical treatments on different types of tissue (Cottet-Rousselle, Ronot, Leverage, & Mayol, 2011; Mattiasson, Friberg, Hansson, Elmér, & Wieloch, 2003). The mitochondrial marker that has been retained as the most pertinent for these studies is membrane potential. Over a long period of time, the mitochondrial potential collapses. To detect these different states, mitochondrial enriched fractions were studied with two mitochondrial membrane potential measurement probes: Tetra Methyl Rhodamine Methyl ester (TMRM) and Rhodamine 123 (Shapiro, 2000). The selection of mitochondria was carried out according to two criteria: their size and specific marking by Nonyl Acridine Orange (NAO) that binds specifically to cardiolipin in the inner membrane of the mitochondria (Mattiasson, 2004) in order to measure the impact of storage at 4 °C over time on the mitochondrial membrane potential.

2. Materials and methods

2.1. Reagents

4-morpholinepropanesulfonic acid (MOPS), bovine serum albumin (BSA), Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), ethylene-bis (oxyethylenenitrilo)tetraacetic acid (EGTA), malate, proteinase type XXIV, Rhodamine 123 (Rh123), succinate, Tetra Methyl Rhodamine Methyl ester (TMRM), Tris (hydroxymethyl)aminomethane (Trizma® base) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonyl Acridine Orange (NAO), Potassium chloride (KCl) and glutamate were acquired from Fisher Labosi (Paris, France). Magnesium chloride (MgCl₂), potassium dihydrogen phosphate (KH₂PO₄) and sucrose were purchased from Acros Organics (Fair Lawn, NJ, USA). Rh123, TMRM and CCCP were prepared in dimethyl sulfoxide (DMSO) purchased from Thermo Scientific (San Diego, CA, USA).

2.2. Fish muscle

European seabass (*D. labrax*) (300–400 g) were sourced from Aquanord sea farm (Gravelines, France), as previously described (Cléach

et al., 2019). Upon arrival at the laboratory, 2 h after death, the fish were immediately filleted. The fillets were stored on ice in a cold room (at 4 °C to prevent the ice from melting too quickly) for seven days and used for experiments every other day for seven days: D0, D1, D3, D5 and D7. The ice was renewed every day. A plastic wrap was used to avoid contact between the fillets and ice or accumulating water.

2.3. Mitochondrial isolation

Mitochondria were isolated from the fillets of different fish. A piece about 0.5 cm³ (500 mg) of red muscle harvested from the medial portion of the lateral fillet line was dissected from the fillets and cut into thin strips. The obtained strips were incubated at 4 °C for 5 min with 2 mL isolation buffer (ISA: 180 mM KCl, 80 mM sucrose, 5 mM MgCl₂, 10 mM Tris, 2 mM EGTA, pH 7.2 at 4 °C) containing 0.2 mg.mL⁻¹ of bacterial proteinase type XXIV. The tissue suspension was poured into a 3 mL glass Potter homogenizer and homogenized for 3 min using a motorized Teflon pestle at 300 rpm. The homogenate was centrifuged at 7,500 g for 5 min. The resulting pellet was resuspended in 1.5 mL isolation buffer containing 2 mg.mL⁻¹ of fatty acids free BSA and homogenized with pestle motor mixer. The homogenate was then centrifuged at 700 g for 5 min. The supernatant was filled to 1.5 mL with isolation buffer and centrifuged at 1,500 g for 5 min. The supernatant was centrifuged again at 7,000 g for 5 min. The mitochondrial pellet obtained was resuspended in the isolation buffer. Protein concentration was determined using the Bio-Rad protein assay kit, derived from the Bradford method, using BSA as a standard. All mitochondrial isolation steps were performed in a cold room at 4 °C. To conduct oxygraphy and flow cytometry studies, the mitochondrial pellet was resuspended in respiration buffer (125 mM KCl, 20 mM MOPS, 10 mM Tris, 10 μM EGTA, 2.5 mM KH₂PO₄, 2 mg.mL⁻¹ fatty acids free BSA, pH 7.2). For flow cytometry measurements, the amount of mitochondria was adjusted to 50,000 events per μL.

2.4. Transmission electron microscopy

The isolated mitochondrial pellets were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, postfixed in 1% osmium tetroxide in the same buffer and stained with 2% uranyl acetate. After acetonitrile dehydration, samples were embedded in epon-like resin (Embed-812). Ultrathin sections (90 nm) were cut using a Leica UC7 ultramicrotome and collected on 150 mesh hexagonal barred copper grids. After staining with 2% uranyl acetate prepared in 50% ethanol, and incubation with a lead citrate solution, sections were observed on a Hitachi H-600 transmission electron microscope equipped with a W electron source (operated at 75 kV) and a side mounted Hamamatsu C4742-95 digital camera.

2.5. Respiratory control ratio

The respiratory control ratio (RCR) of isolated mitochondria was obtained as follows. Oxygraphy (Rank Brothers digital model 10, Cambridge, United Kingdom) was used to monitor the oxygen consumption of isolated mitochondria at 25 °C. Firstly 2.1 mL of respiration buffer were added to the oxygraphic chamber supplemented with a mixture of glutamate (5 mM), malate (2 mM), and succinate (5 mM). Then, mitochondria were added to a final concentration of 0.2 mg/mL. RCR was calculated as the ratio of state 3 to state 4 rates of respiration (n = 3) (Cléach et al., 2019). RCR was evaluated without a probe (control) and with TMRM and Rh123 probes at a concentration of 50 nM.

2.6. Flow cytometry

A flow cytometer (CytoFLEX Beckman Coulter Life Sciences, Paris, France) was used to monitor the fluorescence of TMRM and Rh123 in order to evaluate changes in mitochondrial potential of isolated mitochondria extracted at different storage times from Day 0 to Day 7. For

this manipulation, the “Blue” laser (488 nm) was used to excite the fluorochromes TMRM and Rh123. The fluorescent channel “PE” (585 nm \pm 42 nm) was used to detect the TMRM emitted fluorescence. The fluorescent channel “FITC” (525 nm \pm 40 nm) was used to detect the Rh123 and NAO emitted fluorescence. For each condition, autofluorescence of cells without staining was analyzed. Data were analyzed using Kaluza software (Beckman Coulter Life Sciences). To evaluate isolated mitochondrial yield, NAO (100 nM), which binds to cardiolipin in the inner mitochondrial membrane, was used.

To measure mitochondrial potential, 1 mL of respiration buffer was added at 25 °C to a 5 mL round-bottom polypropylene tube designed for flow cytometry analysis. TMRM or Rh123 (50 nM), without or with substrates (glutamate (5 mM), malate (2 mM) and succinate (5 mM)) were added to the respiratory buffer. For the acquisition, between 1 and 1.6 μ L isolated mitochondria were added in order to record 50,000 events using the 10 μ L.min⁻¹ setting for sample flow rate. In the absence of respiratory substrates, the isolated mitochondria oxidize the undefined endogenous substrates initially present in the mitochondria after extraction. The decoupling agent CCCP (1 μ M final concentration) was then added to the preparation to disrupt the potential, leading to the TMRM or Rh123 output from the mitochondria matrix. The percentage of depolarized mitochondria in the presence of CCCP with and without substrates was the negative control.

2.7. Statistical analysis

The statistical analysis was performed by ANOVA and generated with SPSS 17 software. Changes in percent mitochondrial polarization over time were assessed in triplicate. Data are expressed as mean \pm standard deviation. Unpaired two-sample *t*-tests were used to express the significance of difference ($p < 0.05$) between means, and Levene's test was used to determine the homogeneity of variance.

3. Results

3.1. Electron microscopic analysis of isolated mitochondria

Electron microscopy had a dual purpose. It consisted, on the one hand, in checking the status of purity of the preparation and, on the other, in controlling the status of the mitochondria after extraction (Fig. 1). The vast majority of the isolated mitochondria was quite well preserved. However, differences in enrichment were observed between preparations. The enrichment level on day 4 was higher than on D0 and D7. Mitochondrial extracts showed rough reticulum debris and the presence of various vesicles. However, the size of these vesicles was much smaller ($>0.5 \mu$ m). The presence of myofibrillar debris was easily identifiable on these images (Fig. 1). The size of the mitochondria in the isolate remained fairly homogeneous and varied between 1 and 2 μ m. The size of the mitochondria was fairly homogeneous across the images (Fig. 1A to 1C and 1A' to 1C'), indicating that the mitochondria do not exhibit excessive swelling during the storage period of the fillets at 4 °C. By D0, the mitochondria appeared with well-defined membranes and a well-formed network of crests. On some mitochondria, detachment of the crests revealed intermembrane spaces (to be distinguished from vacuolization) (Fig. 1A and 1A'). On D4, the density of the outer membrane seemed decreased for certain mitochondria. The network of crests sometimes appeared disorganized for some mitochondria. At this stage, densification (in black) could be observed at different locations of the mitochondrial matrix (Fig. 1.B and 1B'). By D7, the mitochondria retained their shape, but the outer membranes and crests were disorganized. Initial vacuolization could be observed. Similarly, the dense granules were a little more visible at D7 (Fig. 1C and 1C').

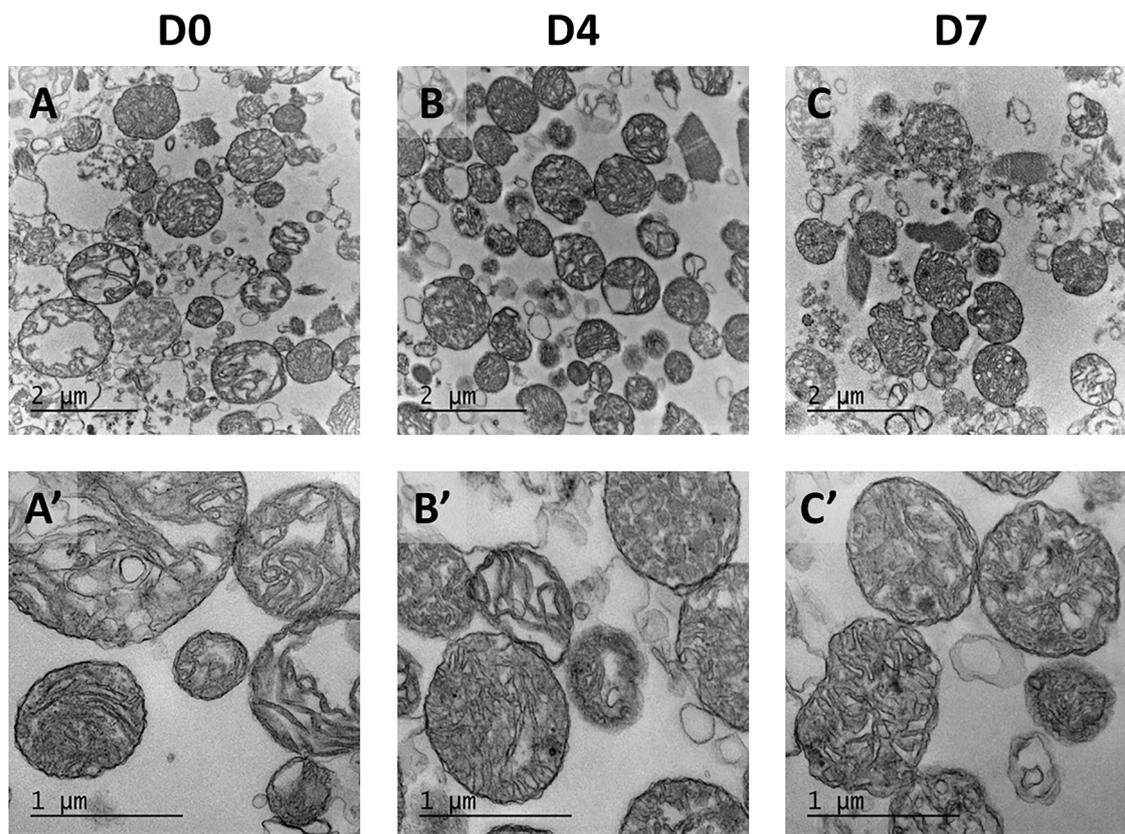


Fig. 1. Electron microscopy of mitochondria isolated from seabass fillets stored at 4 °C. A. Day 0; B. Day 4; C. Day 7 (Magnification X15000). A'. Day 0; B'. Day 4; C'. Day 7 (Magnification X30000).

3.2. Mitochondrial targeting

The heterogeneity of the isolates obtained led to discrimination of mitochondria compared to other cellular debris. A first selection was made according to particles size and structure (Side scatter SSC and Forward scatter FSC analysis) (Fig. 2.A). The gate selected made it possible to focus on the elements of interest in a region called R1, which represented 75.38% of 10,000 events (Fig. 2.A). Events within R1 were plotted for their fluorescence at 525 nm to determine the unstained control sample (Fig. 2.B). Two different regions were identified: R2 (64.88% of R1 events) and R3 (0.07% of R1 events). In this case, the distribution of particles between regions R2 and R3 was 99.9% and 0.1%, respectively (Fig. 2.B). To determine the proportion of mitochondria from the R1 region, the sample was labelled with NAO (Fig. 2.C). In this case, the R2 region represented 9.74% of the R1 events, while the R3 region represented 53.37% of the R1 events. The distribution of particles between the R2 and R3 regions, where particles were NAO-positive, was 15.43% and 84.57%, respectively (Fig. 2.C).

These results confirmed that the R1 region was very largely composed of mitochondria. This region was studied when marking the mitochondria with TMRM and Rh123 to measure the potential at different storage times at 4 °C. NAO and TMRM/Rh123 labeling were not carried out together, due to the proximity of their emission spectra.

3.3. Mitochondrial membrane potential

Mitochondrial activity was studied according to the storage time of the seabass fillets at 4 °C. Changes in the native mitochondrial potential of isolated red muscle mitochondria from seabass fillets stored at 4 °C were monitored over time, from D0 to D7. In order to ensure that the probe concentrations had no impact on mitochondrial respiration, and therefore on mitochondrial potential, the measurement of respiration was carried out in the absence of probes and in the presence of TMRM or Rh123. The presence of these probes at concentrations used for flow cytometry (50 nM) had no impact on respiration (Respiratory Control Ratio: RCR). RCR was determined from isolated mitochondria in the absence of probes and in the presence of TMRM or Rh123 fluorescent probes. The hundred percent was the relative calculation of the RCR obtained without probe ($n = 3, p > 0.05$, Student's *t*-test). TMRM had a percentage of 93.5 ± 13 , while Rh123 had a percentage of 104 ± 17 . These two probes could thus be used to measure potential variations in the isolated mitochondria seabass.

3.3.1. Mitochondrial membrane potential without substrates

The native mitochondrial potential corresponds to the potential of

the mitochondria without the addition of substrates.

3.3.1.1. Measurements of the membrane potential with TMRM and without substrates. The level of TMRM incorporation in isolated mitochondria from seabass fillets was studied at different storage times in the absence of substrates (Fig. 3A). The CCCP indicator at D0 dissipated the mitochondrial potential and showed that the mitochondria fixed the probe. This negative control verified the fluorescence level of fully depolarized mitochondria. At D0, in the presence of CCCP, mitochondria were highly depolarized. Mitochondria located in the R2 region (81.34 %) showed low potential (Fig. 3A). At D0, the level of probe incorporation was elevated: 70.48% of the mitochondria had a high potential (R3 region), compared to 9.68% at low potential (R2 region) (Fig. 3A). The 19.83% (% (R1 - (R2 + R3) events) of undetected events in these two areas corresponded to elements other than the mitochondria. They most likely reflected the percentage of purity of the mitochondrial homogenate (80.17%). The distribution of mitochondria between the R2 and R3 regions was 12.08% and 87.92%, respectively reflecting a very large majority of isolated mitochondria with a high potential. From D0 to D7, the potential of mitochondria isolated from seabass samples gradually decreased over storage time. The percentage of high potential mitochondria decreased from 57.54% at D1 to 15.12% at D7 (Fig. 3A). Conversely, the percentage of low potential mitochondria increased from 22.5% at D1 to 63.41% at D7 (Fig. 3A). At D7, the distribution of mitochondria between the R2 and R3 regions was 80.75% and 19.25%, respectively reflecting a very large majority of isolated mitochondria with low potential. Using TMRM probe in the absence of substrates, the preservation of fish fillets at 4 °C was accompanied by a loss of mitochondrial potential over time.

3.3.1.2. Measurements of membrane potential with Rhodamine 123 and without substrates. The results obtained with TMRM were confirmed with the use of a second potential probe: Rh123 (Fig. 4A). CCCP was also used as a negative control. At D0, in the presence of CCCP, mitochondria were highly depolarized. Mitochondria located in the R2 region (76.92%) showed low potential (Fig. 4A). At D0, the percentage of high-potential mitochondria was high (72.63%), compared with 10.53% of low-potential mitochondria (Fig. 4A). The 16.84% (% (R1 - (R2 + R3) events) of undetected events in these two areas corresponded to elements other than the mitochondria. They reflected the percentage of purity of the mitochondrial homogenate (83.16%). The distribution of mitochondria between the R2 and R3 regions was 12.67% and 87.33%, respectively reflecting a very large majority of isolated mitochondria with a high potential. From D0 to D7, the potential of mitochondria isolated from seabass samples gradually decreased over the storage time.

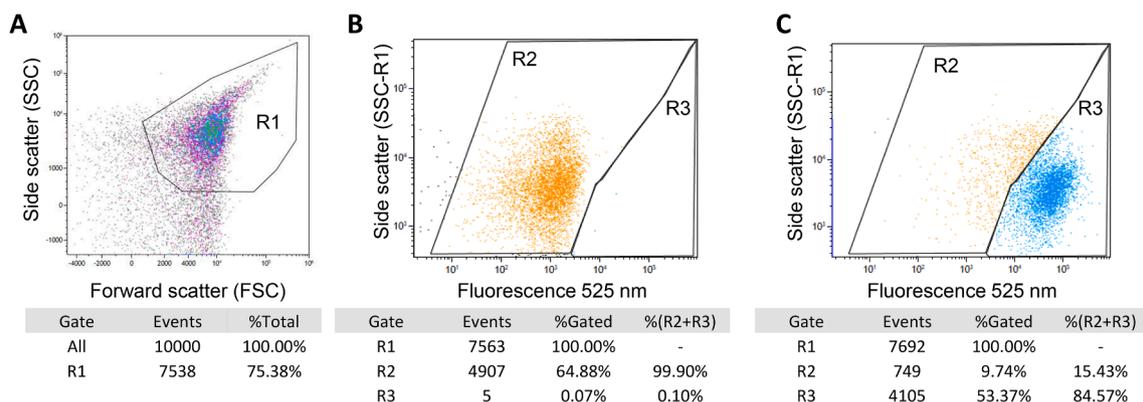
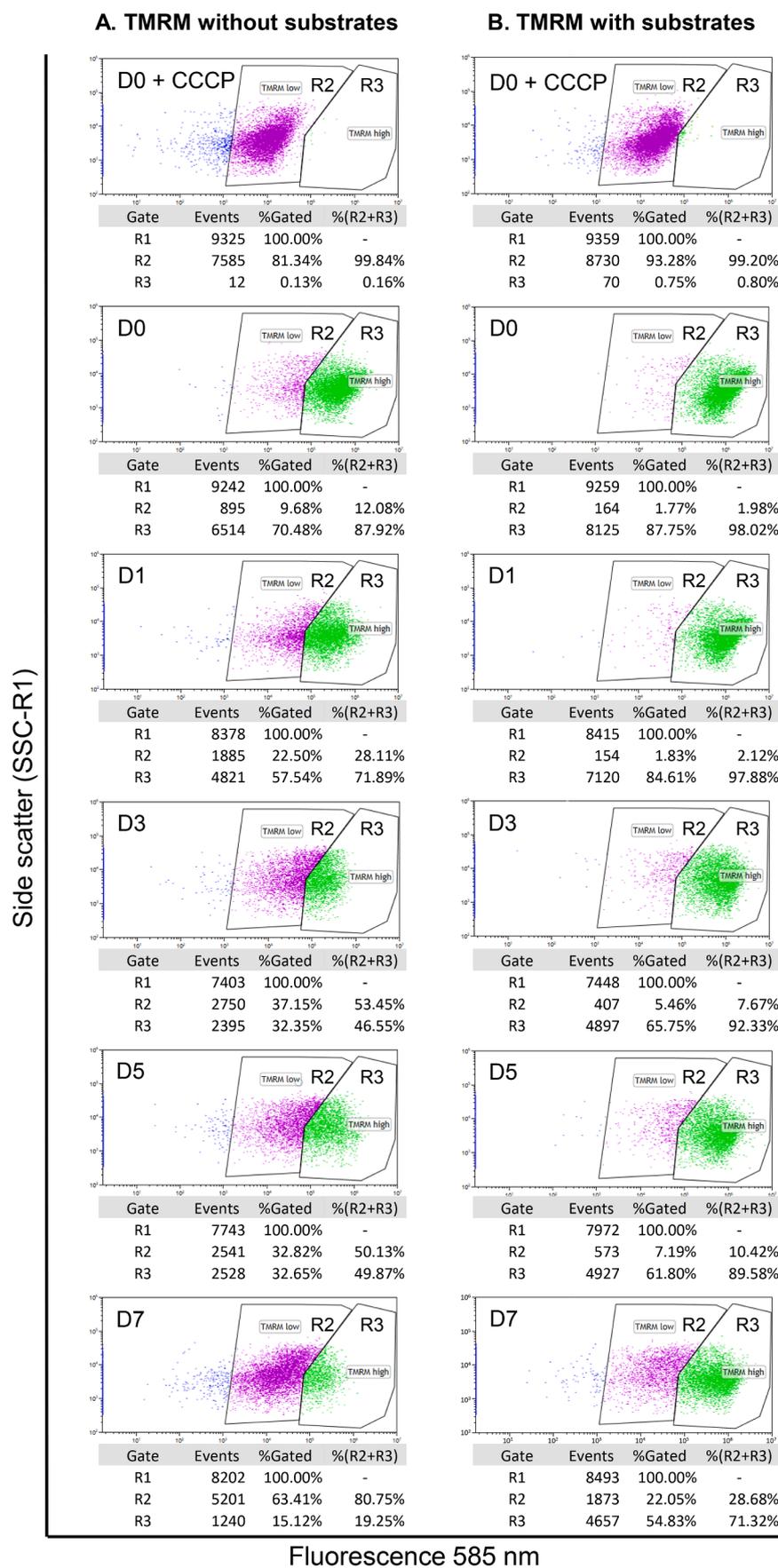


Fig. 2. Isolated mitochondria selection based on size (Forward Scatter: FSC), structure (Side Scatter: SSC) and on selective labeling of mitochondria cardiolipin with 100 nM NAO (Nonyl Acridine Orange). A. Choice of the gate among 10,000 events. Definition of the R1 region and its proportion (%) of the total. B. Fluorescence at 525 nm of the events within R1 for an unstained control sample. Definition of the R2 and R3 regions and their proportion of the R1 region. C. Fluorescence at 525 nm of the events within R1 for a NAO stained sample. Proportion of regions R2 and R3 of the R1 region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



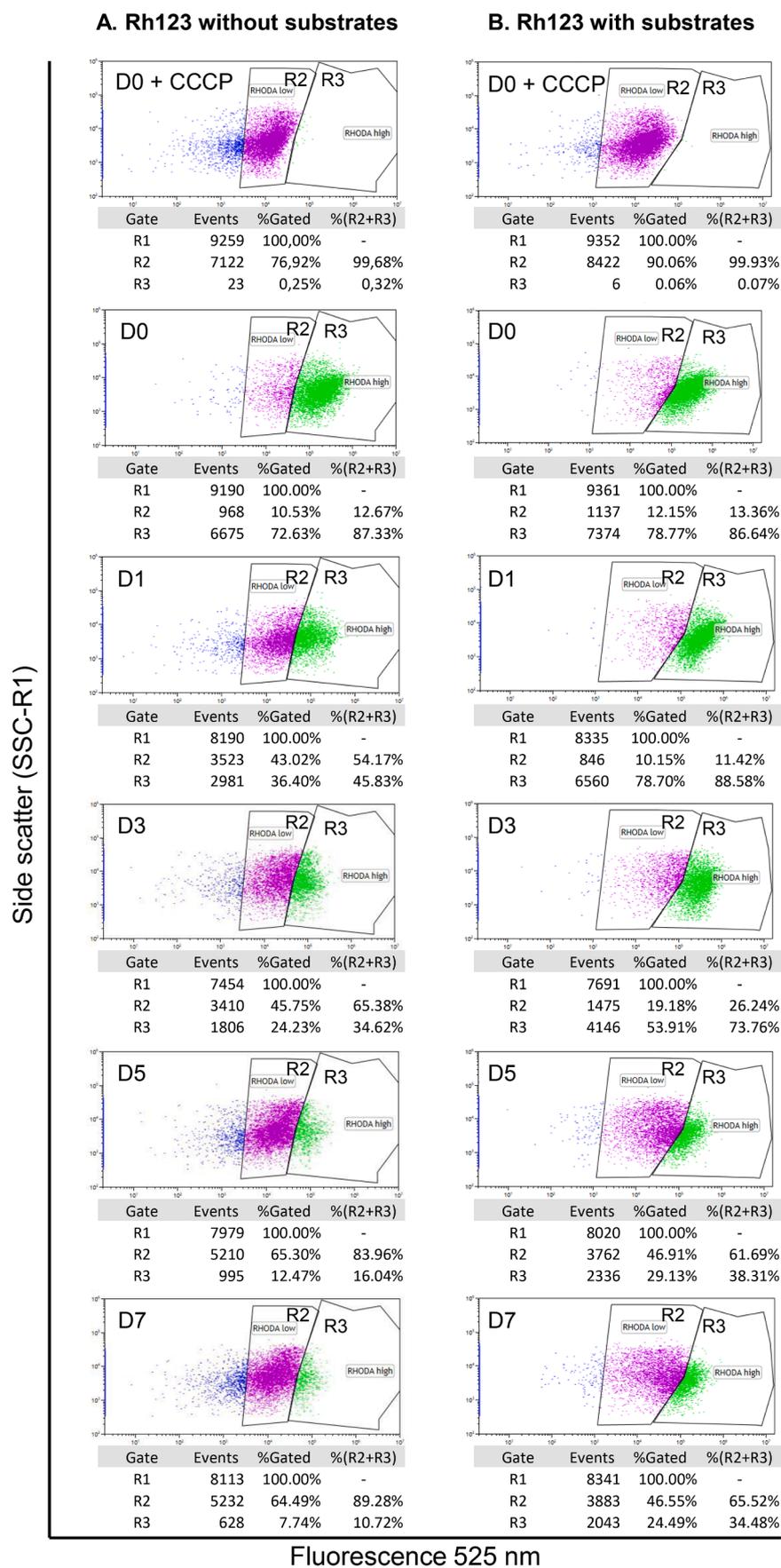


Fig. 4. Measurements of membrane potential with Rhodamine 123 in mitochondria isolated from seabass filets for different storage times (Day 0 at Day 7). A. The Rh123 probe was used at a concentration of 10 nM and fluorescence emission was measured at 525 nm in the absence of substrates (glutamate, malate and succinate). B. The Rh123 probe was used at a concentration of 10 nM and fluorescence emission was measured at 525 nm in the presence of substrates (glutamate, malate and succinate). The negative control was obtained by dissipation of the mitochondrial potential with CCCP (2 μM) at D0. Events were followed between the R1, R2 and R3 regions. The percentages were expressed on the total events of the R1 gate (%Gated). The distribution of events between the R2 and R3 regions was expressed as a percentage of all events related to these two regions: % (R2 + R3).

The percentage of high-potential mitochondria decreased from 36.40% at D1 to 7.74% at D7 (Fig. 4A). Conversely, the percentage of low-potential mitochondria increased from 43.02% at D1 to 64.49% at D7 (Fig. 4A). At D7, the distribution of mitochondria between the R2 and R3 regions was 89.28% and 10.72%, respectively reflecting a very large majority of isolated mitochondria with a low potential. Using the Rh123 probe in the absence of substrates, the preservation of fish fillets at 4 °C was accompanied by a loss of mitochondrial potential over time.

3.3.1.3. Summary of the analysis with fluorescent probes without substrates. These results obtained with each of the two probes showed that discrimination by the size and structure of the mitochondria made it possible to eliminate the contaminants and to measure the physiological status of the mitochondria via their potential. Without substrates, mitochondria isolated from seabass samples rapidly lost their potential over time (Fig. 5A and 5B). Using TMRM or Rh123 probes without substrates, a significant decrease in the percentage of polarized mitochondria was observed from D0 to D7. TMRM was a more sensitive

probe than Rh123 for the measurement of mitochondrial potential over time, especially between D0 and D5. At D7 they reached a very low potential level, close to that of CCCP depolarized mitochondria.

3.3.2. Mitochondrial membrane potential repolarization with substrates

Post-mortem biochemical reactions are numerous and contribute to the production of many metabolites, such as pyruvate (glycolytic pathway). Different types of metabolites are able to constitute substrates for the mitochondrial respiratory chain, such as glutamate, malate and succinate. The availability of these substrates could therefore condition the physiological activity of mitochondria in *post-mortem* tissue. The purpose of adding the substrates was to study the *post-mortem* physiological activity of isolated mitochondria. In this part, we compared the potential of isolated mitochondria using two different probes (TMRM and Rh123) in the presence of substrates (Fig. 3B and 4B).

3.3.2.1. Measurements of the membrane potential with TMRM and with substrates. CCCP was used as a negative control. At D0, in the presence

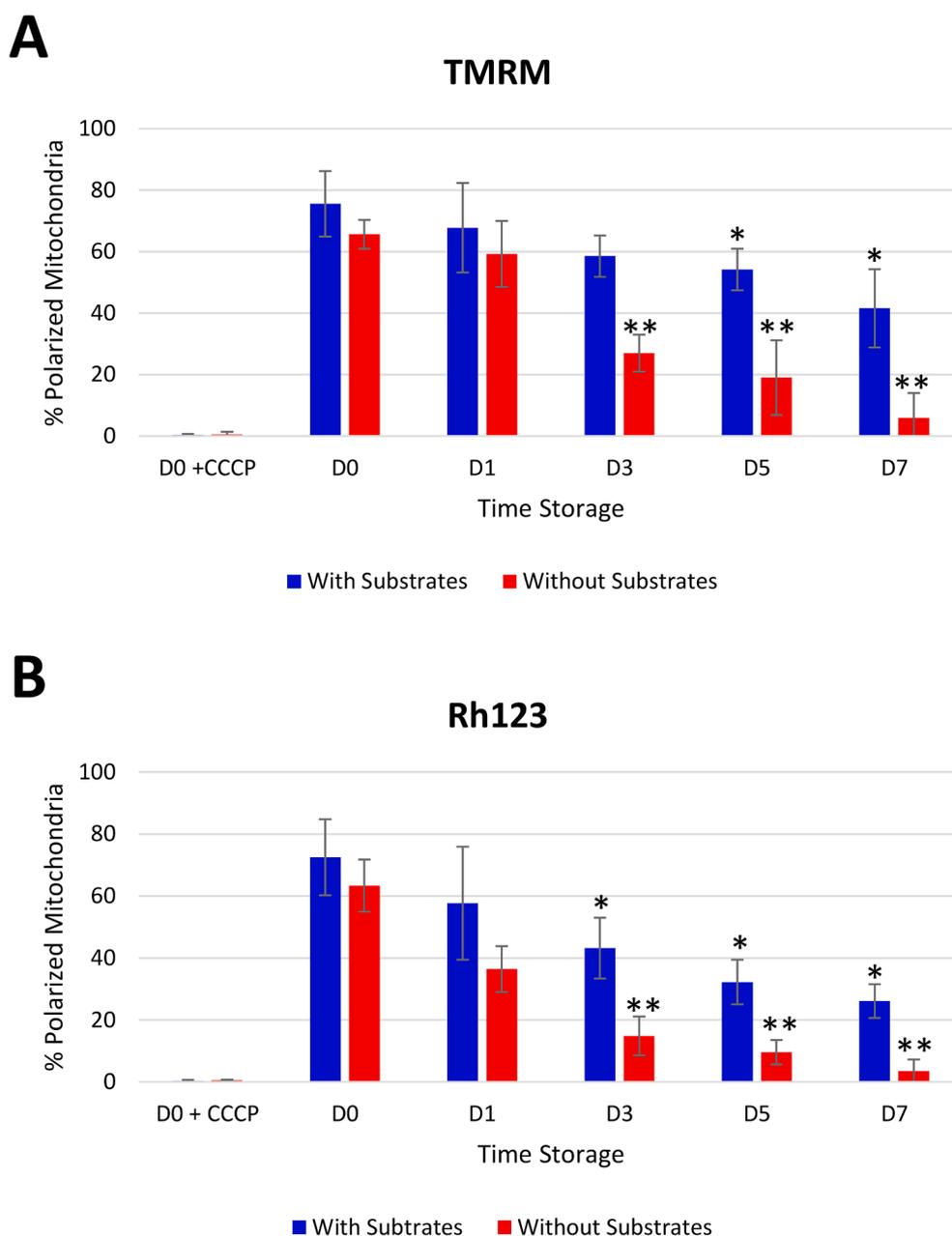


Fig. 5. Percentage of polarized mitochondria evolution in seabass fillets stored at 4 °C for different storage times (Day 0 at Day 7) A. The polarization of the mitochondria was carried out with the TMRM probe used at the concentration of 10 nM and fluorescence emission was measured at 525 nm in the presence and in the absence of substrates (glutamate, malate and succinate) B. The polarization of the mitochondria was carried out with the Rh123 probe used at the concentration of 10 nM and fluorescence emission was measured at 525 nm in the presence and in the absence of substrates (glutamate, malate and succinate) The negative control was obtained by dissipation of the mitochondrial potential with CCCP (2 μM) at D0 Asterisks denote values that are significantly different at different storage times. The *t*-test and Levene's test were carried out on three different storage time measurements in triplicates for each timepoint using the SPSS Statistics 17 program. * $p < 0.05$ versus D0. ** $p < 0.05$ versus "with substrates".

of CCCP, mitochondria were highly depolarized. Mitochondria located in the R2 region (93.28%) showed low potential (Fig. 3B). From D0 to D5, the addition of substrates led to a relatively large increase of the mitochondrial potential (Fig. 3B). The substrate effect was greater as the storage time increased. At D0, the distribution was as follows: 87.75% of mitochondria were at high potential, and 1.77% of mitochondria were at low potential, with a distribution of mitochondria between the R2 and R3 regions of 1.98% and 98.02% respectively, reflecting a very large majority of isolated mitochondria with a high potential (Fig. 3B). At D5, the high/low potential distribution was 61.80%/7.19%. Similarly, at D7, the distribution was as follows: 54.83% of mitochondria were at high potential and 22.05% were at low potential (Fig. 3B). At D7, the distribution of mitochondria between the R2 and R3 regions was 28.68% and 71.32%, respectively showing a greater proportion of high potential mitochondria in the presence of substrates compared to the same time in the absence of substrates (Fig. 4A and B).

3.3.2.2. Measurements of the membrane potential with Rh123 and with substrates. CCCP was used as a negative control. At D0, in the presence of CCCP, mitochondria were highly depolarized. Mitochondria located in the R2 region (90.06%) showed low potential (Fig. 4B). From D0 to D5, the addition of substrates led to a relatively large increase of the mitochondrial potential. The substrate effect was greater as the storage time increased indicating depletion of endogenous substrates throughout time post mortem. At D0, the distribution was as follows: 78.77% of mitochondria were at high potential, and 12.15% were at low potential, with a distribution of mitochondria between the R2 and R3 regions of 13.36% and 86.64%, respectively reflecting a very large majority of isolated mitochondria with a high potential (Fig. 4B). At D5, the high/low potential distribution was 29.13%/46.91%. Similarly, at D7, the distribution was as follows: 24.49% of mitochondria were at high potential, and 46.55% were at low potential (Fig. 4B). At D7, the distribution of mitochondria between the R2 and R3 regions was 65.52% and 34.48%, respectively reflecting a large majority of isolated mitochondria with a high potential with substrates.

3.3.2.3. Summary of the analysis with fluorescent probes with substrates. With substrates, mitochondria isolated from seabass samples have gradually lost their potential over time (Fig. 5A and 5B). Using TMRM or Rh123 probes with substrates, a significant decrease in the percentage of polarized mitochondria was observed from D0 to D7. TMRM was a more sensitive probe than Rh123 for the measurement of mitochondrial potential over time, especially between D0 and D7.

3.3.3. Comparative study between staining mitochondria without or with substrates

The substrates malate, glutamate and succinate largely contributed to repolarize the mitochondria. The effect of the substrates was much lower at D5 and D7 (Fig. 3B and 4B). At these times, a significant proportion of the mitochondria were no longer repolarizable after the addition of substrates. At D7, with the TMRM probe, the addition of substrates induced a high/low distribution of 54.83%/22.05% versus 87.75%/1.77% at D0 (Fig. 3B). The trends were equivalent with Rh123 (Fig. 4B), although the relative percentages of high potential mitochondria were lower. The difference in the percentage of polarized mitochondria with substrates performed on several kinetics ($n = 3$) was very significant for times D3, D5 and D7 (Fig. 5A and 5B). The proportion of non-polarizable mitochondria by the substrates increased rapidly beyond D7 (results not shown), which indicated profound alteration of the mitochondria and more precisely uncoupling of the mitochondrial inner membrane. These results complemented those obtained from previous studies (Cleach et al., 2018; Cléach et al., 2019; Cléach, Soret, Gard, & Lencel, 2020) by showing that the effect of substrates can also provide important information on the physiological status of the mitochondria and thereby the tissue.

4. Discussion

Flow cytometry is a technique used to assess the characteristics of whole cell mitochondria and isolated mitochondria. The interest of working on isolated mitochondria is twofold: it allows mitochondria to be isolated from any tissue, and it provides an idea of the physiological status of the tissue (Lampl, Crum, Davis, Milligan, & Del Gaizo Moore, 2015). On the other hand, flow cytometry of isolated mitochondria can also detect morphological variations and subpopulations of mitochondria (Fuller & Arriaga, 2003). In a previous study, we demonstrated that the freshness of seabream fillets could be assessed by isolated mitochondria activity. We measured the respiration and mitochondrial potential on red muscle isolated mitochondria and correlated the freshness level with loss of mitochondrial potential (Cléach et al., 2020). The originality of this study lies in the fact that the freshness evaluation of fish fillets was measured by flow cytometry of red muscle mitochondria isolated from seabass fillets. These results confirmed the relevance of mitochondrial potential in fish freshness measurement. Mitochondrial potential is a reliable indicator of the physiological status of the cell or tissue. It is altered when tissue is ischemic (lack of oxygen) (Lee & Wei, 2012) and deprived of nutrients (disruption of energy metabolism) (Petit, O'Connor, Grunwald, & Brown, 1990). Mitochondrial potential is also affected when cell integrity is affected (Jayaraman, 2005). Consequently, the mitochondrial potential evaluation of isolated mitochondria reflects the cellular status and tissue damage. In this study, purity of the isolated mitochondria preparation for the electron microscopy analyses showed enrichment in mitochondria, which may vary slightly following extraction. Preserving the size and overall structure of the mitochondria over 7 days allowed them to be selected in the same gate (from D0 to D7). We did not observe any significant changes in the size and structure of the mitochondria over 7 days in the selected population. However, there is still a risk of losing a subpopulation of extraction-sensitive mitochondria. In this study, we probably did not recover all the mitochondria. However, the mitochondria that were isolated gave sufficient information to inform us of the physiological status of the tissue. Additionally, flow cytometry analysis made it possible to pre-select the organelle population of interest (in this case mitochondria) and to dispense with more restrictive purification protocols (Percoll) (Sims & Anderson, 2008). Flow cytometry analysis made it possible to work on small samples (10,000 to 50,000 events) and to focus on mitochondria, eliminating cellular constituents that were not of interest. In the flow cytometric analysis, elements were pre-selected according to their size and structure in order to remove the major contaminants from the mitochondrial homogenate. A second selection was done by specifically labeling the samples with NAO, a reliable marker of mitochondria. The high affinity of NAO for cardiolipins explains its high specificity for mitochondria (J. M. Petit, Maftah, Ratinaud, & Julien, 1992). This verified that 80–85% of the events were selected from the NAO-labelled gate. In the selected gate, the percentage of mitochondria was very high and these samples were therefore a representative image of the mitochondria extracted from the seabass fillets.

4.1. Measurement of mitochondrial potential

Flow cytometry of isolated mitochondria made it possible to measure mitochondrial alterations based on various markers: the measurement of membrane potential loss and the generation of reactive oxygen species (ROS), for example. Numerous studies have shown that the extraction of mitochondria is weakly denaturing, and that the isolated mitochondrial potential measurement from muscle tissue was very close to mitochondria *in situ* (Hosseini, Shaki, Ghazi-K.M., & Pourahmad, 2013). Flow cytometry of isolated mitochondria constitutes an alternative to flow cytometry techniques using cells isolated from tissues (Juan, Cavazzoni, Saez, & O'Connor, 1994; Sung et al., 2010). In toxicology, the effect of drugs on tissue can be measured by analyzing the mitochondrial potential of single mitochondria (Kaufmann et al., 2006). The aging of

tissues has also been evaluated through the loss of mitochondrial potential and the increase in ROS on mitochondria isolated from this tissue (Hagen et al., 1997). The loss of mitochondrial potential could come from membrane permeabilization due to permeability transition pores associated with necrotic and apoptotic processes (Lecoeur et al., 2004) or lipid peroxidation by oxygen free radicals (Paradies et al., 1999). In this study, the potential of mitochondria isolated from red muscle of seabass fillets decreased over time. At D0, the proportion of high potential mitochondria was high. The number of high potential mitochondria decreased over time. At D7, the majority of mitochondria isolated from seabass fillets were at low potential. Beyond D7, the mitochondrial potential continued to decrease and tended towards a zero potential at D9 (supplemental data). It was particularly interesting to observe that red muscle mitochondria manage to maintain potential over this period of time. Mitochondrial potential decreased almost linearly over time, and therefore constitutes an essential marker for measuring the freshness of fish fillets.

4.2. Mitochondria repolarization

Isolated mitochondria lost their potential almost linearly with storage time at 4 °C without and with substrates. Decreased potential is known to attenuate the generation of ROS (Sack, 2006), which can cause irreversible damage. However, the loss of mitochondrial potential could simply be related to the availability of the substrates for the mitochondria. The addition of succinate, glutamate and malate helped to repolarize the mitochondria. Until D5 with TMRM probe, the addition of substrates made it possible to regain the native potential (D1 without substrates). After this time, part of the mitochondria could no longer be polarized by the substrates, resulting in an irreversible loss of mitochondrial potential. The availability of substrates in the tissue therefore appeared to be decisive for the maintenance of mitochondrial activities. Our analysis made it possible to distinguish three levels of freshness. The amount of high potential mitochondria corresponded to ultra-fresh tissue status. Subsequently, mitochondria showed decreased potential and could be repolarized. This corresponded to an intermediate status of freshness. Finally, a third status was observed: the mitochondria were depolarized and were difficult to repolarize. Moreover, the alteration of the potential was greater in white tissue (result not shown) than in red tissue. Therefore, the maintenance of mitochondrial potential could result from higher maintenance of substrates in red tissue than in white tissue. Glycogen reserve and *post-mortem* lactate production is higher in red muscle (Choe et al., 2008). The LDH level is 200 to 240 times higher in red tissue than in white tissue (Yoshida et al., 2007). Therefore, the production of substrates such as pyruvate, able to maintain the mitochondrial potential in *post mortem* conditions, would be greater in red muscle (Yu, Tian, Shao, Li, & Dai, 2019). The maintenance of mitochondrial potential could also be linked to the substrates available in the seabass tissue.

5. Conclusion

Flow cytometric analysis of mitochondria isolated from red muscle of seabass fillets and stored at 4 °C showed mitochondrial potential loss over time. This analysis made it possible to distinguish three levels of freshness: the ultra-fresh level corresponding to fillets where the mitochondria retain high membrane potential; the intermediate freshness level where mitochondria have lost some of their potential but remain repolarizable after the addition of substrates; and lastly, the lower freshness level where mitochondria have lost their potential and are no longer repolarizable by substrates. Flow cytometric analysis of the potential of mitochondria isolated from seabass fillets is a highly reliable method that should be developed to confirm the freshness of fish.

CRedit authorship contribution statement

Méline Soret: Methodology, Resources, Writing – review & editing. **Tiffanie Bouchendhomme:** Resources, Writing – review & editing. **Jérôme Cleach:** Writing – review & editing. **Nathalie Jouy:** Writing – review & editing. **Claire Crola Da Silva:** Writing – review & editing, Methodology. **Anne Devin:** Writing – review & editing, Validation. **Thierry Grard:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Philippe Lencel:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131690>.

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