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► **To cite this version:**

Tiffanie Bouchendhomme, Méline Soret, Thierry Grard, Philippe Lencel. Differentiating between fresh and frozen-thawed fish fillets by muscle fibre permeability measurement. *Food Control*, 2023, 147, pp.109567. 10.1016/j.foodcont.2022.109567. hal-03922533

**HAL Id: hal-03922533**

**<https://ulco.hal.science/hal-03922533>**

Submitted on 4 Jan 2023

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1 **Differentiating between fresh and frozen-thawed fish fillets by muscle fibre permeability**  
2 **measurement**

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13 **Highlights**

14 Mitochondrial properties were used to discriminate fresh and frozen-thawed fillets

15 Permeability of muscle fibres and mitochondrial membranes to NADH was measured

16 Two approaches were used to measure permeability: spectrophotometry and oxygraphy

17 Frozen-thawed fibres were more permeable to NADH and less sensitive to alamethicin

18 **Abstract**

19 There is no comprehensive method for differentiating between fresh and frozen-thawed fish  
20 fillets. This is an ongoing problem, particularly in relation to regulations. In this study, we  
21 showed the relevance of using the properties of mitochondria to discriminate fresh fish fillets  
22 from frozen-thawed fish fillets. The use of red muscle fibres of Gilthead sea bream allowed us  
23 to leave mitochondria in their physiological environment and to avoid possible alterations of  
24 mitochondrial membranes during isolation steps. The impact of freezing on fillets was  
25 evaluated by measuring the permeability of fibres and mitochondrial membranes to  
26 nicotinamide adenine dinucleotide + hydrogen (NADH). NADH permeability of fresh fillet  
27 fibres stored at 4°C was compared to the permeability of fibres extracted from frozen-thawed  
28 fillets. Two approaches were used to measure permeability changes: a spectrophotometric  
29 method that measured consumption of NADH by complex I, and an oxygraphic approach that  
30 measured stimulation of O<sub>2</sub> consumption by NADH. Fibres from frozen-thawed fillets were  
31 more permeable to NADH and were less sensitive to the permeabilizer alamethicin. The

32 sensitivity of this method allowed us to clearly detect red muscle fibres from frozen-thawed  
33 fish versus fresh fish fillets.

34 **Key words:** oxygraphy, spectrophotometry, mitochondria, NADH, permeabilization, muscle  
35 fibres

## 36 **1. Introduction**

37 Many species of fish are caught at considerable distances from consumption sites. As a result,  
38 storage of aquatic products can include a freezing stage. These products are generally  
39 intended to supply the frozen food market (Costello & Ovando, 2019). Consumption of frozen  
40 products has tended to decrease in recent years, although it increased again in 2020 with the  
41 COVID-19 pandemic. Detecting possible supply of frozen-thawed seafood to the fresh fish  
42 market is a real problem. The development of molecular tools to detect fraud within the  
43 aquatic products industry is becoming increasingly relevant (Sone et al., 2019).

44 Given that freezing leads to the formation of ice crystals that cause irreversible damage to  
45 plasma and intracytoplasmic membranes (Li, 2018), we built on this finding in previous  
46 studies. We measured the impact of fish fillet freezing on the permeabilization of  
47 mitochondria isolated from red muscle fibres (Bouchendhomme et al., 2022). Freezing of  
48 aquatic products leads to changes in mitochondrial membranes which become far more  
49 permeable. This permeability can be measured in different ways: via the release of matrix  
50 proteins or metabolites, or via permeability to certain substrates (Sileikyte et al., 2010).  
51 Increased permeability to nicotinamide adenine dinucleotide + hydrogen (NADH) is  
52 associated with mitochondrial membrane alteration related to freezing-thaw shock (Kroemer  
53 et al., 2007).

54 Isolation of mitochondria requires a series of extraction steps. In this study, our objective was  
55 to reduce analysis time by using muscle fibres directly, without a mitochondria extraction  
56 step. This technique has been used for many years to explore mitochondrial functions for  
57 medical purposes, including the detection of myopathies, neurodegenerative diseases and  
58 cancer (Kuznetsov et al., 2022). Muscle fibres are usually mechanically dissociated and  
59 permeabilized with saponin or digitonin to make substrates and inhibitors accessible to  
60 mitochondria (Kuznetsov et al., 2008). This treatment is used to enable exploration of  
61 mitochondrial functions while preserving their state and their interactions with other  
62 organelles (Picard et al., 2010, 2011). The use of muscle fibres is particularly well adapted to  
63 the discrimination of frozen-thawed aquatic products from fresh ones. In this context, freezing  
64 shock induces permeabilization of plasma and intracytoplasmic membranes making the fibres

65 and mitochondria more sensitive to substrates and to certain inhibitors present in the  
66 extracellular medium (Stéphenne et al., 2007). Due to the high concentration of mitochondria  
67 in red muscle, the amount of fibres required for the analysis is low.

68 Evaluation of mitochondrial function in muscle fibres has been used to measure differences in  
69 meat quality in pork and beef products (Ramos et al., 2020; Werner et al., 2010). Use of  
70 muscle fibres allows for the measurement of differences in post-mortem respiratory activities  
71 in various breeds. Permeabilized fibres have also been used to link post-mortem  
72 mitochondrial activities to meat quality (England et al., 2018; Ramos et al., 2021). In this  
73 study, we aimed to use isolated red muscle fibres to explore mitochondrial functions that  
74 might be affected by freezing processes (Tolstorebrov et al., 2016). The chosen model  
75 allowed us to measure the sensitivity of muscle fibres to NADH with or without  
76 permeabilization (Mayevsky & Barbiro-Michaely, 2009; Mayevsky & Rogatsky, 2007). Our  
77 study focused on the action of freezing on plasma and mitochondrial membranes by  
78 measuring the sensitivity of mitochondria to NADH (Batandier et al., 2004). Two techniques  
79 were used: the first was to measure consumption of NADH on fresh and frozen-thawed fibres  
80 by a spectrophotometric method. The second was to measure O<sub>2</sub> consumption by oxygraphy  
81 on fresh and frozen-thawed fibres, in the presence of NADH. The permeabilization level of  
82 the membranes was normalized by using a permeabilizing agent, alamethicin (Matic et al.,  
83 2005). In this way, it was possible to assess levels of membrane permeabilization by  
84 measuring the level of NADH consumption and the level of O<sub>2</sub> respiration. The objective was  
85 to study whether permeabilization induced by freezing remained significant compared to  
86 permeabilization induced by autolysis processes (Pegg, 2010), linked to the storage of  
87 products at low temperatures (2–4°C) (Diop et al., 2016).

## 88 **2. Materials and methods**

### 89 *2.1 Biological material*

90 Gilthead seabream (*Sparus aurata*) (300–450 g) were sourced from Aquanord-Ictus sea farm  
91 (Gravelines, France), as previously described (Cléach, *et al.*, 2019). Filets were stored in a  
92 cold room (+4°C) on ice. The core temperature of fishes was 2°C. Ice renewal was carried out  
93 every day. Fillets intended for freezing were frozen at -40°C at D0 in order to minimize  
94 damage to cell structures (Bao et al., 2021). Analyses were performed at day 1, day 4 and day  
95 8 for fresh fillets and for frozen-thawed fish after 9 days in the freezer. The thawing phase  
96 was carried out in a cold room at 4°C. To avoid contact between fillets and ice, plastic  
97 wrapping was used.

98 *2.2 Reagents*

99 Bovine serum albumin (BSA), ethylene glycol-bis (2 amino-ethylether)-N,N,N',N'-tetraacetic  
 100 acid (EGTA), sucrose, potassium chloride (KCl), Tris(hydroxymethyl)aminomethane  
 101 (Trizma<sup>®</sup> base), 4-morpholinepropanesulfonic acid (MOPS), alamethicin,  $\beta$ -nicotinamide  
 102 adenine dinucleotide (NADH), decylubiquinone and cytochrome c were purchased from  
 103 Sigma-Aldrich (St. Louis, MO, USA). Magnesium chloride (MgCl<sub>2</sub>) and potassium phosphate  
 104 (KH<sub>2</sub>PO<sub>4</sub>) were purchased from Acros Organics (Morris Plains, NJ, USA). Alamethicin was  
 105 prepared in methanol purchased from Fisher Scientific (Loughborough, UK). Cytochrome c  
 106 was prepared in distilled water. Decylubiquinone was prepared in dimethyl sulfoxide  
 107 (DMSO) purchased from Thermo Scientific (San Diego, CA, USA).

108 *2.3 Fibre preparation*

109 Fibres originated from red muscle of Gilthead seabream. In brief, 10 mg of fibres were taken  
 110 from the dorsal part of the red muscle. Fibres were dilacerated using scalpels and with the  
 111 help of very fine tweezers under a binocular magnifying glass. Under these conditions, muscle  
 112 fibres were separated from conjunctive tissue. Fibres were then rinsed with respiratory buffer.  
 113 The respiratory buffer was composed of KCl 125 mM, MOPS 20 mM, Tris 10 mM, KH<sub>2</sub>PO<sub>4</sub>  
 114 2.5 mM, MgCl<sub>2</sub> 2.5 mM, EGTA 10  $\mu$ M and BSA 2 mg.mL<sup>-1</sup>. Fibres were stored in respiratory  
 115 buffer until analysis. Measurements on the fibres were carried out in a time of 90 min or less.  
 116 This handling time included isolation and analysis time.

117 *2.4 NADH consumption by spectrophotometry*

118 NADH consumption was measured by spectrophotometry (UV vis spectrophotometer, UV-  
 119 1280, Shimadzu Europa GmbH, Duisburg, Germany). The spectrophotometric tank contained  
 120 1 mL of the respiratory medium, 5 mg of fibres, cytochrome c (10  $\mu$ M) and decylubiquinone  
 121 (0.1 mM). Rotenone (2.5  $\mu$ M) was used as a control. Alamethicin (5  $\mu$ M) was added to  
 122 permeabilize the fibres. NADH was used at 0.1 mM final concentration in all tanks. The zero  
 123 calibration was done without NADH and reading was performed at 340 nm. Measurements  
 124 were taken at t=0s, t=100s, t=200s and t=300s. Between each measurement, tanks were placed  
 125 on a vibrating stirrer at 150 rpm (Titramax 100, Heidolph instruments GmbH, Schwabach,  
 126 Germany).

127 The specific activity (SA) was calculated from the law of Beer-Lambert:

128 
$$SA = \left( \frac{\left( \frac{AA}{\epsilon l} \right) / \Delta T}{\text{quantities of fibres}} \right) / 1000 \text{ nmol of NADH} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ of wet mass.}$$

129  $\varepsilon = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$

130 *2.5 O<sub>2</sub> consumption by oxygraphy*

131 O<sub>2</sub> consumption was measured using an oxygraph O-2k (Oroboros Instruments, Innsbruck,  
132 Austria). The 100 % calibration was done when the chamber was open in the presence of a  
133 maximum amount of O<sub>2</sub>. The 0 % calibration was done in the closed chamber and with  
134 addition of dithionite. Before addition of the fibres, cytochrome c (10 µM) and  
135 decylubiquinone (0.1 mM) were added. After 3 minutes, the fibres were added and the  
136 chamber was closed. NADH (1 mM) was added, then alamethicin (5 µM). The experiment  
137 was performed at +25°C.

138 O<sub>2</sub> consumption was calculated using this formula:

$$O_2 \text{ consumption} = \frac{(O_2 \text{ consumption with substrate} - O_2 \text{ consumption without substrate})}{\text{quantities of fibres}} \times 60$$

139 O<sub>2</sub> consumption was expressed in pmol of O<sub>2</sub>.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass.

140 *2.6 Discrimination threshold*

141 A ratio can be calculated using this formula:

$$\text{ratio} = \frac{\text{consumption with alamethicin}}{\text{consumption without alamethicin}}$$

142 This ratio was used to determine a discrimination threshold value. Below a certain value,  
143 frozen-thawed fillets were found.

144 *2.7 Statistical analysis*

145 Statistical analyses and graphs were generated with XLSTAT 2022.1.1. Each experiment was  
146 repeated 4 times. Data were expressed as means ± standard deviation. A *t*-test was used to  
147 express the significance of difference ( $p < 0.05$ ) between the different means.

148 **3. Results**

149 *3.1 Measurement of NADH consumption by spectrophotometry*

150 Rates of NADH consumption (in nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass) on isolated fresh  
151 fillet fibres kept at 4°C (F) and on isolated frozen-thawed fillet fibres (T) and in the presence  
152 or absence of the permeabilizer alamethicin (+/-) were studied (Fig. 1).

153 NADH consumption on fresh fibres was low. It was 779.96 nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet  
154 mass. The addition of alamethicin (FD+) led to a considerable permeabilization effect, which

155 resulted in a significant increase in NADH consumption by fresh fibres. Consumption reached  
156 2161.27 nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass. Consumption of NADH by frozen-thawed  
157 fibres without the addition of alamethicin was high and was 3152.86 nmol of NADH.min<sup>-1</sup>  
158 .mg<sup>-1</sup> of wet mass, whereas the addition of alamethicin did not lead to any increased  
159 consumption of NADH. Frozen-thawed fibres had a higher NADH consumption rate and were  
160 not sensitive to the action of the permeabilizer (alamethicin), unlike fresh fibres.

### 161 *3.2 Effect of fillet storage time on NADH consumption*

162 Rates of NADH consumption (in nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass) on isolated fresh  
163 fillet fibres stored at 4°C and for different storage times (1 day, 4 days and 8 days) were  
164 studied (Fig. 2).

165 Storage time had no significant effects on NADH consumption by fresh fibres. NADH  
166 consumption decreased from 779.86 nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass at D1 to 476.53  
167 nmol at D4, and to 967.68 nmol at D8. The storage time at 4°C did not seem to have any  
168 influence on the permeabilization of fibres by alamethicin. At D1, the consumption of NADH  
169 in the presence of alamethicin was 2161.27 nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass. It was  
170 2271.98 nmol and 2276.8 nmol of NADH.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass for the times D4 and D8,  
171 respectively. Regardless of the storage time, alamethicin had the same effect on the  
172 permeabilization of fibres and therefore on the consumption of NADH.

### 173 *3.3 Effect of freezing on O<sub>2</sub> consumption by oxygraphy*

174 O<sub>2</sub> concentrations in the oxygen chamber containing 10 mg of isolated fresh fillet fibres and  
175 the rates of O<sub>2</sub> consumption by these fibres were monitored over time (Fig. 3A). Addition of  
176 isolated fibres from a fresh fillet resulted in low consumption of O<sub>2</sub> (less than 25 pmol.s<sup>-1</sup>.mL<sup>-1</sup>  
177 ). After addition of NADH, a slight increase in O<sub>2</sub> consumption was observed; the plateau  
178 indicated a value of 135 pmol.s<sup>-1</sup>.mL<sup>-1</sup> which demonstrated slight permeabilization of the  
179 mitochondria. After addition of the permeabilizer (alamethicin), a significant increase in O<sub>2</sub>  
180 consumption was observed, which was 355 pmol.s<sup>-1</sup>.mL<sup>-1</sup>. This increase showed that fresh  
181 fibres were sensitive to permeabilization; NADH was therefore able to significantly activate  
182 respiration (i.e., O<sub>2</sub> consumption).

183 O<sub>2</sub> concentrations in the oxygen chamber containing 10 mg of isolated frozen-thawed fillet  
184 fibres and the rates of O<sub>2</sub> consumption by these fibres were monitored over time (Fig. 3B).  
185 Addition of isolated fibres from a frozen-thawed fillet resulted in low consumption of O<sub>2</sub> (less  
186 than 20 pmol.s<sup>-1</sup>.mL<sup>-1</sup>). After addition of NADH, a significant increase in O<sub>2</sub> consumption was  
187 observed. The rate of NADH consumption increased without any real plateau effect. It

188 reached about  $180 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$  before the addition of alamethicin which induced greater  
189 permeabilization of mitochondria. After addition of the permeabilizer (alamethicin), we did  
190 not observe a significant increase in  $\text{O}_2$  consumption. The lack of alamethicin effect showed  
191 that fibres from frozen-thawed fillet were not very sensitive to permeabilization. The rate of  
192  $\text{O}_2$  consumption on fibres isolated from frozen-thawed fillet was about two times lower than  
193 for fibres isolated from fresh fillet.

#### 194 *3.4 Effect of fillet storage time on $\text{O}_2$ consumption*

195  $\text{O}_2$  consumption by the fibres as a function of storage time of fish fillets and type of packaging  
196 (fresh or frozen-thawed) is shown in Fig. 4. The rate of  $\text{O}_2$  consumption was expressed in  
197  $\text{pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass and 4 oxygraphs were generated for each condition.  $\text{O}_2$   
198 consumption by isolated fresh fish fibres decreased over time from  $1373.79 \text{ pmol of O}_2\cdot\text{min}^{-1}$   
199  $\cdot\text{mg}^{-1}$  of wet mass at D1 to  $243.72 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass at D8. This showed an  
200 alteration of the respiratory chain complexes which resulted in decreased  $\text{O}_2$  consumption for  
201 an identical stimulation with NADH. Contrary to the spectrophotometric method,  $\text{O}_2$   
202 consumption by the fibres isolated from frozen-thawed fish was not higher than the fibres  
203 isolated from fresh fillets. It was  $1373.79 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass for the fresh  
204 fibres at D1 versus  $1234.08 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass for the frozen-thawed fibres.  
205 Measuring  $\text{O}_2$  consumption of isolated fibres exposed to NADH therefore did not appear to be  
206 sufficient to discriminate between fresh from frozen-thawed fish.

207 For fibres of fresh fillet, we observed an alamethicin effect at all 3 storage times (D1, D4 and  
208 D8). Alamethicin increased NADH permeability of the isolated fresh fillet fibres, which  
209 resulted in increased  $\text{O}_2$  consumption.  $\text{O}_2$  consumption of the fibres in the presence of  
210 alamethicin decreased over time from  $4608.75 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass at D1 to  
211  $891.06 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass at D8. This represents a decrease of 80.66 % and  
212 reflects the fact that respiratory chain efficiency decreased with storage time of the fillets at  
213  $4^\circ\text{C}$ . For fibres of frozen-thawed fillet, there was no significant alamethicin effect.  $\text{O}_2$   
214 consumption was  $1234.08 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass on fibres without alamethicin,  
215 and  $1848.09 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass in the presence of alamethicin. On the 4 trials  
216 performed, this difference was not significant. Freezing led to sufficient permeabilization not  
217 to be amplified by a permeabilizer (alamethicin). We can also note lower  $\text{O}_2$  consumption of  
218 frozen-thawed fillet fibres compared to fresh fillet fibres in the presence of alamethicin.  
219 Consumption decreased from  $4608.75 \text{ pmol of O}_2\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of wet mass for FD1+ to



220 1848.09 pmol of O<sub>2</sub>.min<sup>-1</sup>.mg<sup>-1</sup> of wet mass for T+. Freezing destabilized the activity of the  
221 respiratory chain complexes.

### 222 3.5 Ratio determination to discriminate fish quality (frozen-thawed fillets)

223 An alamethicin effect was present in both methods, spectrophotometric and oxygraphic. This  
224 is why we calculated in this part a ratio  $R = (\text{Activity in presence of alamethicin} / \text{Activity in}$   
225  $\text{absence of alamethicin})$ .

226 In Figure 5A, we present the ratios obtained for the spectrophotometric method as a function  
227 of the storage time of fillets at 4°C and for different packaging modes (fresh and frozen-  
228 thawed). The R ratio for fresh aquatic products was around 2.5–3. The ratio calculated for  
229 frozen-thawed aquatic products was around 1. Using the statistical data, a ratio of 1.5 emerged  
230 for this method. Fresh products could be defined as products with an R ratio > 1.5, frozen-  
231 thawed products as products with an R ratio < 1.5.

232 In Figure 5B, we present the ratios obtained for the oxygraphic method as a function of the  
233 storage time of fillets at 4°C and for different packaging modes (fresh and frozen-thawed).  
234 Ratios were calculated as  $R = (\text{O}_2 \text{ consumption rate in the presence of alamethicin expressed}$   
235  $\text{in pmol of O}_2\text{.min}^{-1}\text{.mg}^{-1} / \text{O}_2 \text{ consumption rate in the absence of alamethicin})$ . The ratio for  
236 fresh products was higher than 3. The calculated ratio for frozen-thawed products was around  
237 1.5. Using the statistical data, a ratio of 2 emerged for this method. Fresh products could be  
238 defined as products with an R ratio > 2, frozen-thawed products as products with an R ratio <  
239 2.

## 240 4. Discussion

241 Changes in membrane permeability associated or not with increased NADH consumption  
242 have been demonstrated by different methodological approaches. These studies based on  
243 fluorescence spectroscopic approaches (Karoui et al., 2021), infrared spectroscopy (Chang et  
244 al., 2020; Cheng et al., 2013; Kamruzzaman et al., 2015), Raman spectroscopy (Herrero,  
245 2008), nuclear magnetic resonance spectroscopy (Cheng et al., 2013), or hyperspectral  
246 imaging (Xu & Sun, 2017) allow the measurement of the profound changes induced during  
247 the frozen/thawed step. Freezing causes profound membrane alterations that lead to  
248 permeabilization of plasma and intracytoplasmic membranes. Plasma and mitochondrial  
249 membranes are naturally impermeable to NADH (Ying, 2006), but freezing physically alters  
250 the membranes making the respiratory enzyme complexes accessible to NADH. In a previous  
251 study, we showed that measuring the NADH permeabilization of isolated mitochondria makes

252 it possible to discriminate between fresh and frozen-thawed seafood products. The use of  
253 muscle fibres allows us to avoid all the extraction steps, but brings an additional level of  
254 complexity insofar as conditions are more heterogeneous (diffusion of NADH in the fibres)  
255 and NADH can be associated with numerous sarcoplasmic enzymatic reactions (Picard et al.,  
256 2012).

#### 257 *4.1 Frozen-thawed fish fillet detection by the spectrophotometric approach*

258 In this study, we showed the effect of freezing at  $-40^{\circ}\text{C}$  on the rupture of plasma and  
259 mitochondrial membranes. Consumption of NADH by the fibres was measured by  
260 spectrophotometry at 340 nm. On fibres isolated from fresh fillets (from D1 to D8), NADH  
261 consumption was low. Consumption of NADH was not attributable to consumption by the  
262 mitochondria because it was insensitive to rotenone (data not shown). In the fresh state, it has  
263 been reported that plasma and mitochondrial membranes remain intact and consumption of  
264 NADH is therefore low and not attributable to the mitochondria (Schantz & Henriksson,  
265 1987). Additionally, consumption of NADH by isolated fresh fish fillet fibres did not increase  
266 with the storage time of the fillets at  $4^{\circ}\text{C}$ . This showed that the autolysis that could occur on  
267 the fibres and that could make NADH accessible to the cytosol did not increase its  
268 consumption. In a previous study, we demonstrated autolysis on a fish fillet by release of  
269 lactate dehydrogenase over several days of storage at  $4^{\circ}\text{C}$  (Diop et al., 2016). We can  
270 hypothesize that this autolysis could make the fibres more permeable to NADH but not the  
271 mitochondria. Artificial permeabilization by alamethicin leads to a significant increase in  
272 NADH consumption by the fibres and thus to permeabilization of the plasma and  
273 mitochondrial membranes (Matic et al., 2005). Here, consumption of NADH was indeed  
274 attributable to the mitochondria because it was able to be blocked by rotenone. Therefore, the  
275 basal consumption by mitochondria on isolated fresh fillet fibres remained low due to the low  
276 level of permeabilization of plasma and mitochondrial membranes. NADH consumption on  
277 fresh fillet fibres was able to be stimulated by alamethicin. This permeabilizer acted on  
278 plasma and mitochondrial membranes, making mitochondria sensitive to NADH. Contrary to  
279 what we had observed on isolated mitochondria, consumption of NADH by isolated fibres  
280 from fresh fillet and in the presence of alamethicin, was not affected by the storage time at  
281  $4^{\circ}\text{C}$ . This was an argument in favour of using fibres to avoid possible denaturation during  
282 extraction processes, especially for mitochondria isolated from fillets that have been stored  
283 longer at  $4^{\circ}\text{C}$  (Zamzami et al., 2007).

284 The relevance of using fibre permeabilization with NADH as a freezing marker was justified  
285 by two important experimental elements. The first was that freezing shock induced a very  
286 significant increase in NADH consumption by the fibres. Freezing leads to a rupture of  
287 plasma and mitochondrial membranes, making complex I accessible to NADH (Yamada et  
288 al., 2020). Blocking of complex I by rotenone leads to a very significant decrease in NADH  
289 consumption by the fibres, suggesting that this consumption is indeed associated with  
290 mitochondria (N. Li et al., 2003). Freezing shock thus permeabilized the plasma and  
291 mitochondrial membranes. The second experimental argument was the absence of a  
292 permeabilizing effect of alamethicin on NADH consumption by isolated frozen-thawed fillet  
293 fibres. The permeabilizing action of freezing on plasma and mitochondrial membranes was  
294 sufficiently strong that no permeabilizing effect of alamethicin on isolated frozen-thawed  
295 fillet fibres was able to be observed (Carraro & Bernardi, 2020). The action of freezing  
296 resulted in maximum NADH consumption by the fibres.

297 The spectrophotometric approach was based on the consumption of NADH by complex I of  
298 mitochondria. The storage of aquatic products at low temperatures (freezing) over relatively  
299 long periods of time could lead to oxidation phenomena. The main site of production of  
300 superoxide compounds, involved in oxidation reactions, is complex I (Mazat et al., 2020).  
301 This production could lead to the oxidation of lipids and peripheral proteins and contribute to  
302 a loss of activity of complex I and meat quality. Therefore, measuring the rate of NADH  
303 consumption by the fibres cannot be an objective factor of alteration. This rate should be  
304 normalized to the rate of NADH consumption by the fibres after addition of alamethicin. This  
305 ratio makes it possible to take into account possible losses of activity of complex I linked to  
306 the storage time at 4°C or to the storage time in frozen state. This ratio allowed us to  
307 determine whether the fibres were sensitive or insensitive to permeabilization (alamethicin).  
308 Fresh aquatic products were defined as products with an R ratio >1.5 and frozen-thawed  
309 aquatic products were defined as products with an R ratio < 1.5.

#### 310 *4.2 Frozen-thawed fish fillets detection by the oxygraphic approach*

311 The oxygraphic method can be considered more specific to mitochondria since O<sub>2</sub>  
312 consumption is directly proportional to NADH consumption by the respiratory chains. O<sub>2</sub>  
313 consumption by fibres isolated from fresh fillet and exposed to NADH tended to decrease  
314 during the time the fillet was kept at 4°C. This result was somewhat surprising but was not  
315 attributable to the change in permeability of the fibres to NADH, but rather related to a loss of  
316 activity of the respiratory chains (Larosa & Remacle, 2018). By permeabilizing the fibres

317 (using alamethicin), we observed a significant decrease in O<sub>2</sub> consumption with the storage  
318 time of the fillets at 4°C. This result showed that the activity of enzyme complexes within the  
319 respiratory chain tended to decrease with storage time. The same result was also observed in a  
320 previous study on mitochondria isolated from fresh fillets (Bouchendhomme et al., 2022).  
321 These results show that measuring increased plasma and mitochondrial membrane  
322 permeability by NADH may be partially biased by a loss of respiratory chain activity.

323 O<sub>2</sub> consumption by isolated fresh fillet fibres was able to be stimulated by alamethicin. As  
324 with the spectrophotometric method, alamethicin acted on the plasma and mitochondrial  
325 membranes and allowed the diffusion of NADH into the mitochondria, activating O<sub>2</sub>  
326 consumption. The activity of the respiratory chains strongly decreased with the storage time,  
327 but the alamethicin effect was observed for all times (D1, D4 and D8).

328 For the oxygraphic method, freezing did not result in a significant increase in O<sub>2</sub> consumption  
329 of the fibres, whereas it induced an alteration of the plasma and mitochondrial membranes.  
330 Why did this happen? Part of the explanation came from the observation of O<sub>2</sub> consumption  
331 of the fibres in the presence of alamethicin. This consumption also decreased substantially  
332 and was close to the O<sub>2</sub> consumption without alamethicin. Therefore, like for fresh fibres, O<sub>2</sub>  
333 consumption by fibres isolated from frozen-thawed fillets stimulated by the increased  
334 permeability of fibres to NADH was partly compensated for by a loss of activity of the  
335 respiratory chains. The respiratory chains seem to be sensitive to the freezing process  
336 (Stéphenne et al., 2007). Therefore, direct O<sub>2</sub> consumption on NADH cannot be an indicator  
337 of freezing. On the contrary, on fibres from frozen-thawed fillets, no alamethicin effect was  
338 observed. It has been shown that freezing causes maximum permeabilization of the fibres  
339 which become insensitive to the effect of the permeabilizer (D. Li et al., 2018; Rasmusson et  
340 al., 2022).

341 As for the spectrophotometric approach, we noted that the direct effect of NADH on O<sub>2</sub>  
342 consumption by the fibres cannot be used as a marker of membrane alteration and therefore of  
343 freezing. It was necessary to reduce this activity to the maximum activity that was obtained in  
344 the presence of alamethicin. Ratio R was defined as the ratio between O<sub>2</sub> consumption  
345 without alamethicin / O<sub>2</sub> consumption in the presence of alamethicin. Calculating this ratio  
346 also allowed us to avoid losses of respiratory chain activity linked to storage at 4°C or to the  
347 storage time in frozen state. Determining the state of the product (fresh or frozen-thawed) is  
348 linked to the sensitivity of the fibres to the permeabilizer alamethicin. If the R ratio is > 2, the  
349 product can be defined as fresh, and if the R ratio is < 2, the product can be defined as frozen-  
350 thawed.

## 351 **5. Conclusion**

352 This study confirmed the relevance of using mitochondria as a frozen-thawed marker. The use  
353 of fish muscle fibres allowed us to avoid the isolation steps of mitochondria and this makes it  
354 possible to obtain results in a much shorter time. Freezing-thawing led to permeabilization of  
355 the plasma and mitochondrial membranes. NADH was then able to access complex I and the  
356 respiratory chains to activate O<sub>2</sub> consumption. Fibres isolated from fresh fillets were sensitive  
357 to permeabilization with alamethicin, resulting in increased permeability to NADH. Fibres  
358 isolated from frozen-thawed fillets were insensitive to the action of alamethicin. This  
359 approach yielded a rapid result on the preservation state of the Gilthead seabream. To  
360 generalize this test to other aquatic products, it would be useful to extend this study to the  
361 white muscle of fish fillets.

## 362 **Declaration of interest**

363 None

## 364 **Acknowledgements and funding**

365 Tiffanie Bouchendhomme would like to thank the Hauts-de-France regional council and  
366 ULCO for their financial support of her PhD studies. The authors would like to thank Philippe  
367 PASDOIS for his scientific and technical advice. This work was funded by the French  
368 government, Ifremer and the Hauts-de-France region in the framework of the CPER 2021-  
369 2027 IDEAL project.

## 370 **Figure legends**

371 **Figure 1:** Relative specific activity of red muscle fibres from fresh and frozen-thawed  
372 Gilthead seabream fillet.

373 F-: Red muscle fibres from fresh fillet without alamethicin

374 F+: Red muscle fibres from fresh fillet with alamethicin

375 T-: Red muscle fibres from frozen-thawed fillet kept at -40°C without alamethicin

376 T+: Red muscle fibres from frozen-thawed fillet kept at -40°C with alamethicin

377 **Figure 2:** Measurement of NADH consumption by spectrophotometry in red muscle fibres  
378 from fresh Gilthead seabream fillet storage at 4°C.

379 FD1-: Red muscle fibres from fresh fillet Day 1 without alamethicin

380 FD1+: Red muscle fibres from fresh fillet Day 1 with alamethicin

381 FD4-: Red muscle fibres from fresh fillet Day 4 without alamethicin

382 FD4+: Red muscle fibres from fresh fillet Day 4 with alamethicin  
 383 FD8-: Red muscle fibres from fresh fillet Day 8 without alamethicin  
 384 FD8+: Red muscle fibres from fresh fillet Day 8 with alamethicin  
 385 Red muscle fibres were incubated with alamethicin (+) or without alamethicin (-)  
 386 Asterisks denote values that are statistically significant  
 387 A *t*-test was performed ( $p < 0.05$ ,  $n = 4$ )

388 **Figure 3:** O<sub>2</sub> consumption of Gilthead seabream red muscle fibres by oxygraphy  
 389 Oxygraph traces (blue curve) and first derivate (red curve) are represented at different storage  
 390 conditions.  
 391 A. Oxygraph of red muscle fibres from D1 fresh fillet  
 392 B. Oxygraph of red muscle fibres from frozen-thawed fillet  
 393 The run was carried out in the presence of cytochrome C (10 µM) and decylubiquinone (0.1  
 394 mM). After the addition of red muscle fibres (10 mg), NADH (0.1 mM) and alamethicin (2.5  
 395 µM) were added to the incubation chamber.

396 **Figure 4:** Relative O<sub>2</sub> consumption of red muscle fibres from fresh and frozen-thawed  
 397 Gilthead seabream fillets.  
 398 FD1-: Red muscle fibres from fresh fillet Day 1 without alamethicin  
 399 FD1+: Red muscle fibres from fresh fillet Day 1 with alamethicin  
 400 FD4-: Red muscle fibres from fresh fillet Day 4 without alamethicin  
 401 FD4+: Red muscle fibres from fresh fillet Day 4 with alamethicin  
 402 FD8-: Red muscle fibres from fresh fillet Day 8 without alamethicin  
 403 FD8+: Red muscle fibres from fresh fillet Day 8 with alamethicin  
 404 TD8-: Red muscle fibres from frozen-thawed fillets kept for 8 days at -40°C without  
 405 alamethicin  
 406 TD8+: Red muscle fibres from frozen-thawed fillets kept for 8 days at -40°C with alamethicin  
 407 Red muscle fibres were incubated with alamethicin (+) or without alamethicin (-)  
 408 Asterisks denote values that are statistically significant  
 409 A *t*-test was performed ( $p < 0.05$ ,  $n = 4$ )

410 **Figure 5:** Relative R ratio from fresh and frozen-thawed Gilthead seabream fillets.  
 411 For both methods, the R ratio is determined by the ratio between the activity in the presence  
 412 of alamethicin and the activity in the absence of alamethicin ( $R = (\text{activity with alamethicin}) /$   
 413  $(\text{activity without alamethicin})$ ).

414 A. R ratio of red muscle fibres by the spectrophotometric method

415 B. R ratio of red muscle fibres by the oxygraphic method

416 The red line defines the ratio that appears to be significant to distinguish fresh from frozen-  
417 thawed fillets.

418 A *t*-test was performed ( $p < 0.05$ ,  $n = 4$ )

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