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### ► To cite this version:

Alexandre Dehaut, Marlène Chollet, Philippe Jacques, Frédéric Krzewinski, Assi N'Guessan, et al.. Use of degenerate primers to detect and quantify torA gene harbored by specific spoilage organisms of fish. Food Micro - 24th International ICFMH Conference, Sep 2014, Nantes, France. anses-03889676

**HAL Id: anses-03889676**

**<https://anses.hal.science/anses-03889676v1>**

Submitted on 8 Dec 2022

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# Use of degenerate primers to detect and quantify *torA* gene harbored by specific spoilage organisms of fish

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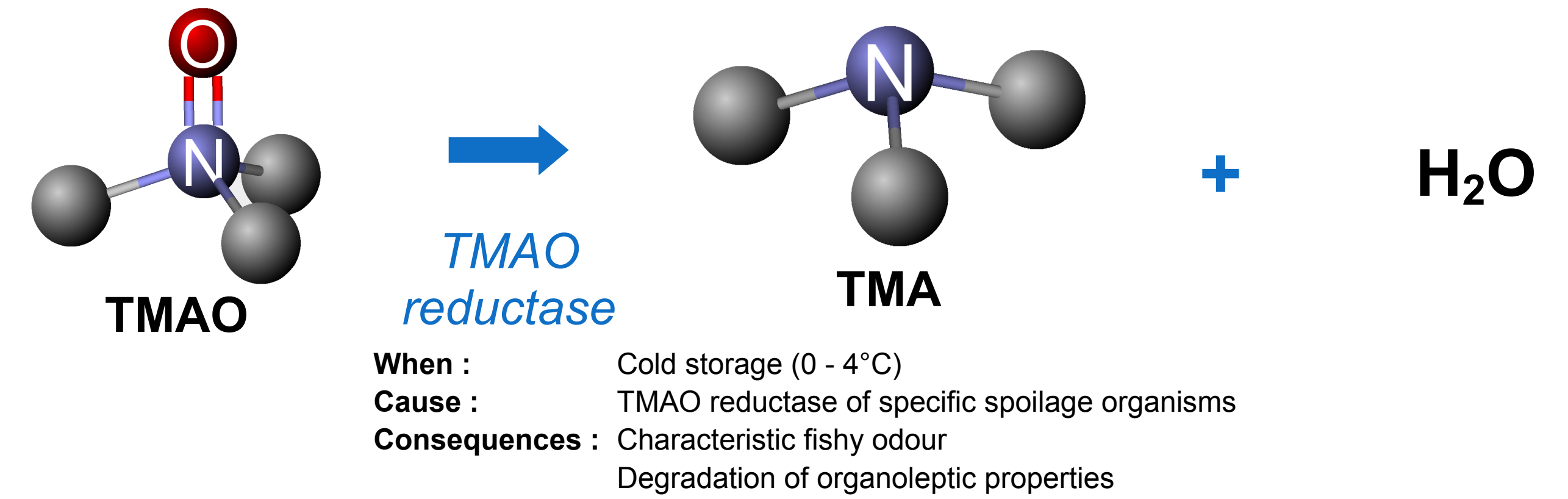


## Introduction

→ Fish is a highly perishable food matrix. To date, most of the developed techniques are more rejection tool than monitoring methods. Moreover, these techniques become reliable once the early freshness step is exceeded.

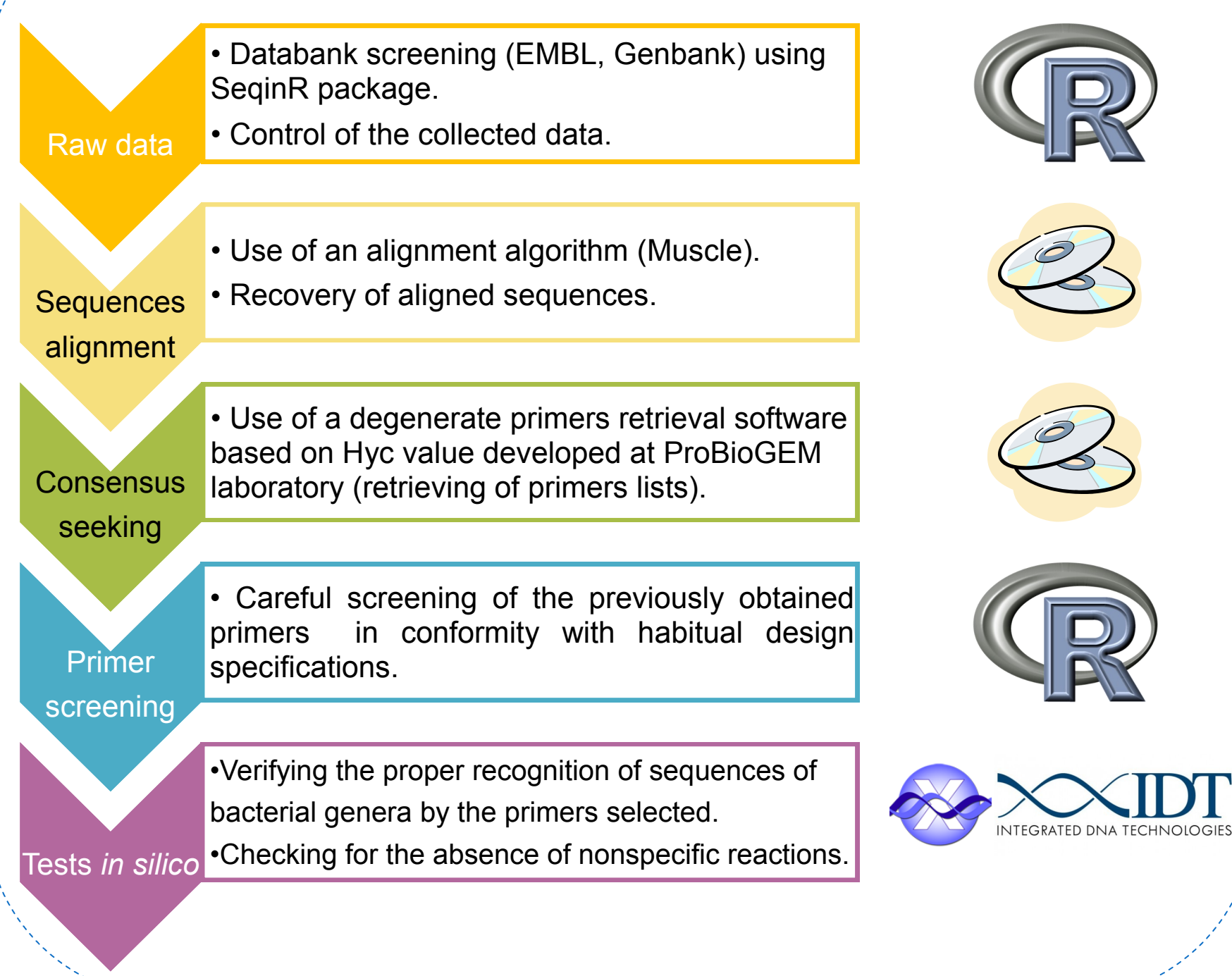
→ The major cause of the freshness decay is due to the development of spoilage flora called specific spoilage organisms (SSO). During fish spoilage SSO reduce trimethylamine N-oxide (TMAO), present in the fish flesh, thanks to an enzyme called TMAO reductase encoded by *torA* gene. This reaction leads to the production of trimethylamine (TMA), part of the total volatile basic nitrogen (TVB-N), responsible of the specific, fishy off-odour.

→ The aim of this work was to develop and apply a molecular technique (qPCR) targeting *torA* gene harboured by SSO and use this technique for the monitoring of fish spoilage.



## Materials & Methods

### → Primers design and *in silico* characterization



### → DNA extractions

. DNA extraction achieved thanks to Qiagen Blood & Tissue kit for both pure culture and spoiling fish flesh.  
 . Quantity and quality controlled with Denovix DS-11.

### → *In vitro* characterization of the primers

. Selection of promising primer pairs.  
 . Efficiency tests & optimization of amplifications conditions.  
 . Specificity tests.

### → Methods used for the monitoring of fish shelf-life

. Study of modified-atmosphere packed whiting fillets (*Merlangius merlangus*), stored at +1.0°C and prepared as follow:



. 3 fish analyzed each day (D1, D3, D6, D8, D10, D13, D15) for:



## Results

- . More than 750 forward and reverse primers were suggested by ProBioGEM software for each genera *Vibrio*, *Photobacterium* & *Shewanella*, with Hyc values comprised between 1 and 32.
- . Screening algorithm allowed to discard more than 88% of above mentioned primers. AmpliX permitted to find 13600 potential amplicons from the 3 genera. 6 pairs were retained to further *in vitro* characterization.
- . A pair VF239 x VR353 gave positive results with a single band at 420 pb.

Table 1: Efficiency test for VF239xVR353 according to qPCR parameters

	Slope <sup>a</sup>	[Primers] 1 µM		[Primers] 0.5 µM	
		62°C	64°C	62°C	64°C
<i>P. phosphoreum</i> DSM2167	Intercept <sup>b</sup>	-3.512	-3.539	-3.679	-3.834
	R <sup>2</sup>	0.998	0.998	0.999	0.998
	Efficiency (%) <sup>b</sup>	92.6	91.7	87.0	82.3
	Slope <sup>a</sup>	-3.542	-3.631	-3.615	-3.813
<i>P. damselae</i> CIP100540	Intercept <sup>b</sup>	37.8	38.5	38.5	40.0
	R <sup>2</sup>	0.999	0.999	0.999	0.996
	Efficiency (%) <sup>b</sup>	91.6	88.5	89.1	82.9
	Slope <sup>a</sup>	-3.512	-3.774	-3.802	-4.030
<i>V. vulnificus</i> CIP 75.4	Intercept <sup>b</sup>	37.1	38.5	39.2	41.2
	R <sup>2</sup>	0.993	0.999	0.999	0.994
	Efficiency (%) <sup>b</sup>	92.6	84.1	83.3	77.4
	Slope <sup>a</sup>	-3.503	-3.810	-3.738	-3.979
<i>V. alginolyticus</i> CIP 101888	Intercept <sup>b</sup>	36.7	38.5	38.1	40.1
	R <sup>2</sup>	0.996	0.999	0.999	0.995
	Efficiency (%) <sup>b</sup>	93.0	83.0	85.1	78.4

<sup>a</sup> Values were obtained from four replicates on 7 log DNA dilutions ranging from 15 µg mL<sup>-1</sup> to 15 pg mL<sup>-1</sup> of template DNA in the tube. <sup>b</sup> Efficiency was calculated according to the formula given as follows: Efficiency = 10<sup>(-1/Slope)</sup> - 1

. Primers pair VF239 x VR353 amplified the expected PCR product for two species of both *Photobacterium* and *Vibrio* genera.

. Impact of both annealing temperature and primers concentration on pair efficiency for all tested strains.

. Best amplification efficiency, higher than 90%, recorded at 62°C with primers concentration of 1 µM for the four studied species.

Table 2: Results of the specificity study

Strains	Mean Cq <sup>a</sup>	Band at the expected size <sup>b</sup>	Aspecific bands	Sequencing results <sup>c</sup>
<i>V. cholerae</i> CIP 106974	10.98 ± 0.02	Yes	No	100 % identity with <i>V. cholerae</i> IEC224 <i>torA</i> gene
<i>V. parahaemolyticus</i> CNRVC 010089	11.77 ± 0.03	Yes	No	99% identity with <i>V. parahaemolyticus</i> VP2007-007 <i>torA</i> gene
<i>V. mimicus</i> CIP 101888	10.93 ± 0.01	Yes	No	99% identity with <i>V. mimicus</i> VMS73 <i>torA</i> gene
<i>V. fuvialis</i> CIP 103355	13.98 ± 0.01	Yes	No	96 % identity with <i>V. fuvialis</i> NCTC 11218 <i>torA</i> gene
<i>V. metschnikovi</i> CIP 69.14	34.22 ± 0.05	Yes	Yes	No sequencing
<i>V. harveyi</i> CIP 103192	11.94 ± 0.01	Yes	No	99% identity with <i>V. harveyi</i> ATCC BAA-1116 <i>torA</i> gene
<i>B. subtilis</i> ATCC 104354	29.55 ± 0.06	Yes	Yes	No sequencing
<i>V. furtisii</i> CIP 102972	12.92 ± 0.01	Yes	No	98 % identity with <i>V. furtisii</i> NCTC 11218 <i>torA</i> gene
<i>V. nigriluchitoides</i> CIP 103195	11.44 ± 0.01	Yes	No	98 % identity with <i>V. cholerae</i> IEC224 <i>torA</i> gene
<i>S. putrefaciens</i> DSM 6067	36.52 ± 0.11	No	Yes	No sequencing
<i>S. ballica</i> 44LSABSMS1SHW	30.89 ± 0.01	Yes	Yes	90 % identity with <i>S. ballica</i> BA 175 <i>torA</i> gene
<i>B. subtilis</i> ATCC 6633	34.18 ± 0.53	Yes	No	75% identity with <i>V. vulnificus</i> CMCP6 <i>torA</i> gene
<i>S. aureus</i> ATCC 25923	37.54 ± 0.73	No	No	No sequencing
<i>S. epidermidis</i> ATCC 12228	36.65 ± 0.93	No	No	No sequencing
<i>E. coli</i> ATCC 25922	29.68 ± 0.15	No	Yes	No sequencing
<i>S. enterica</i> ATCC 13076	34.91 ± 1.00	Yes	Yes	98 % identity with <i>S. enterica</i> CT18 hypothetical protein
<i>C. maltaromaticum</i>	37.83 ± 0.36	No	No	No sequencing
<i>C. divergens</i>	34.92 ± 0.25	Yes	No	96 % identity with <i>V. vulnificus</i> CMCP6 <i>torA</i> gene
<i>C. gilleni</i>	31.16 ± 0.20	Yes	Yes	93 % identity with <i>S. ballica</i> BA175 <i>torA</i> gene
<i>S. proteomaculans</i>	32.82 ± 0.01	No	Yes	No sequencing
<i>P. agglomerans</i>	17.84 ± 0.22	No	Yes	No sequencing
<i>P. fluorescens</i>	34.01 ± 0.09	No	Yes	No sequencing

<sup>a</sup> Mean of Cq values recorded for duplicate measures, for template DNA at 15 µg mL<sup>-1</sup>. <sup>b</sup> Existence of a band close to the expected size of 420 bp. <sup>c</sup> Percentage of identification achieved thanks to BLAST; "no sequencing" is either marked for strains displaying no band at the expected size, or for band not sequenced because of the presence of another band close to.

. PCR product obtained for a wide range of *Vibrio* species.

. *Shewanella* species displayed aspecific amplification patterns.

. Surprisingly *B. subtilis*, *C. divergens* & *C. gilleni* displayed a PCR product similar to *torA* gene sequences of *V. vulnificus* and *S. ballica*.

### → Reference method assessing microbial burden

. Total Viable Count led to non-workable results: firstly increase from day 1 to day 6, then decrease until day 15.

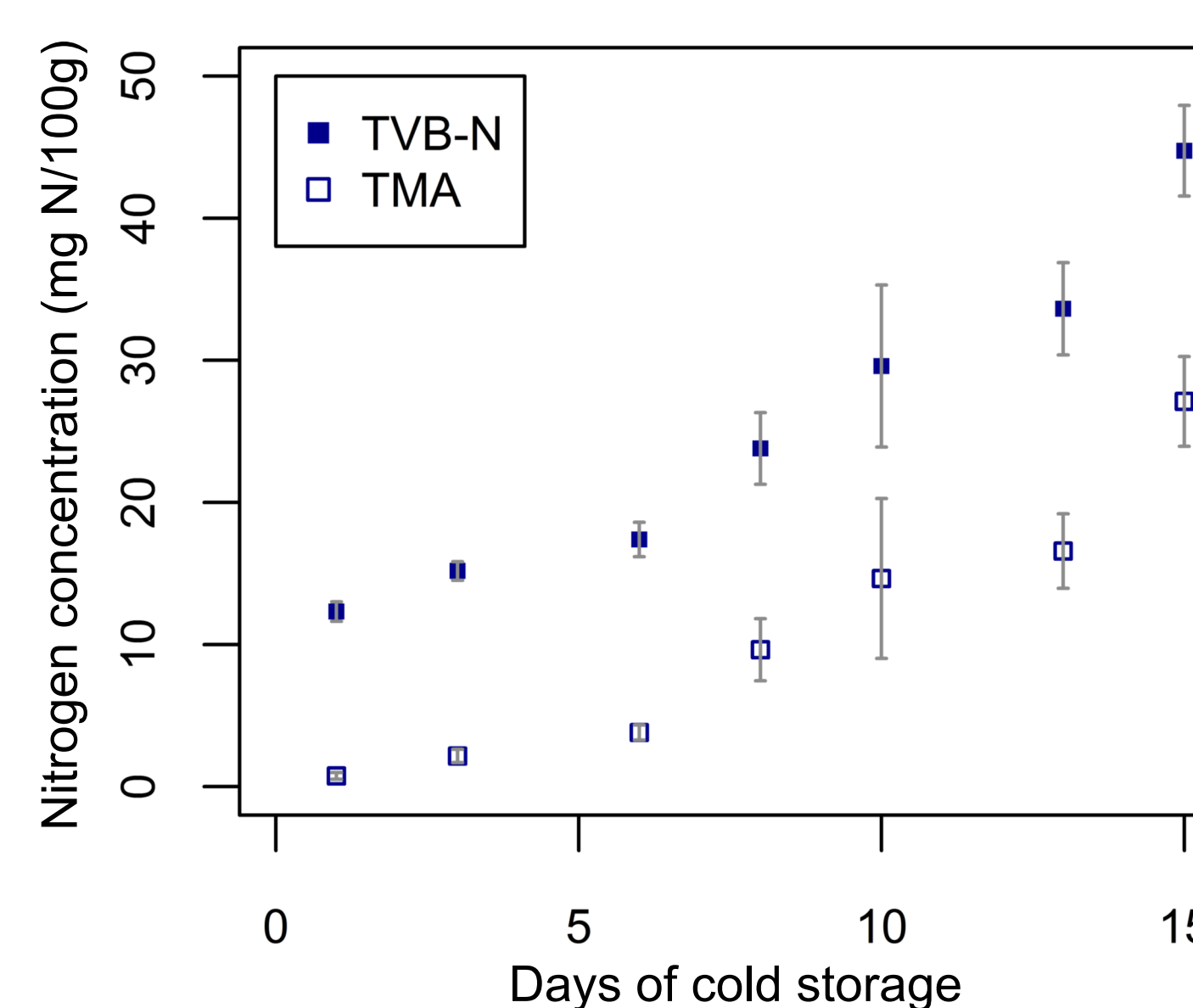


Figure 1: Average TVB-N & TMA values (n=6) per day throughout the shelf-life.

### → Reference method assessing freshness grade throughout shelf-life (Fig. 1)

. Slight increase of both TMA & TVB-N values until day 6, then increase marked by more variability among fish tested.

. Based on TVB-N values, fillets considered as spoiled at the neighbourhood of day 10.

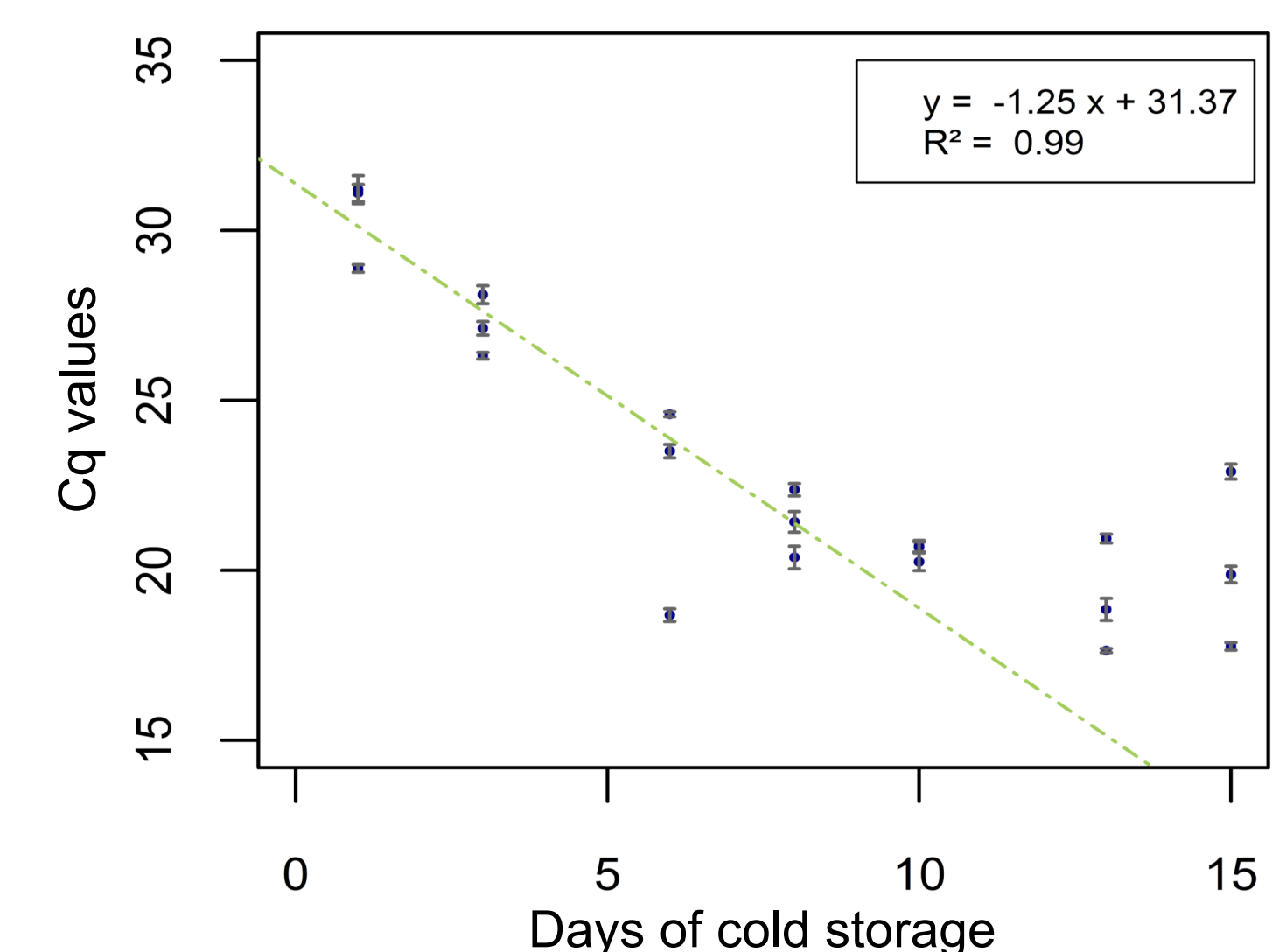


Figure 2: Mean Cq values (n=4) calculated per fish throughout the study. Green dashed line corresponds to the linearity zone from day 1 to day 8.

### → Shelf-life assessment by qPCR (Fig. 2)

. Clear decrease of Cq value, linear (R<sup>2</sup> = 0.99) for the 8 first days and stabilization beyond.

. Results anti-correlated with TVB-N (-0.86) and TMA (-0.81) from day 1 to day 13.

. Sequencing of PCR products range from 76% (at day 3) to 90% identity (after day 6) with *P. phosphoreum* DSM 2167 *torA* gene.

. Probable outgrowth of *P. phosphoreum* throughout spoilage.

## Conclusions

- First results described herein are encouraging to develop a new monitoring approach, suitable for the early states of fish freshness.
- Rapid and sensitive method for the freshness estimation of more than 30 fish within a day, easily applicable in food analysis laboratories.
- Highly probable confirmation of *P. phosphoreum* outgrowth, previously described as SSO of modified-atmosphere packed chilled marine fish.
- Bioinformatics approach using different software allowed to secure primers design, from sequence retrieval to *in silico* primers characterization.
- VF239 x VR 353 primers pair displayed the best efficiency for *Vibrio* and *Photobacterium* species (at 62°C for primers concentrations of 1 µM).

- Implementation of sensory analysis adapted to the matrix, should give better insights in the rejection limits of fish.
- Addition of controls: samples process control, sample negative control, environmental control, IAC has to be carry out.
- This method has to be tested on other shelf-life studies, other storage conditions and other fish species.

## Perspectives