

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

DEHAUT Alexandre¹, CHOLLET Marlène², JACQUES Philippe², KRZEWINSKI Frédéric³, N'GUESSAN Assi⁴, KOL Ossarath³, GRARD Thierry⁵, BRISABOIS Anne¹ & DUFLOS Guillaume¹

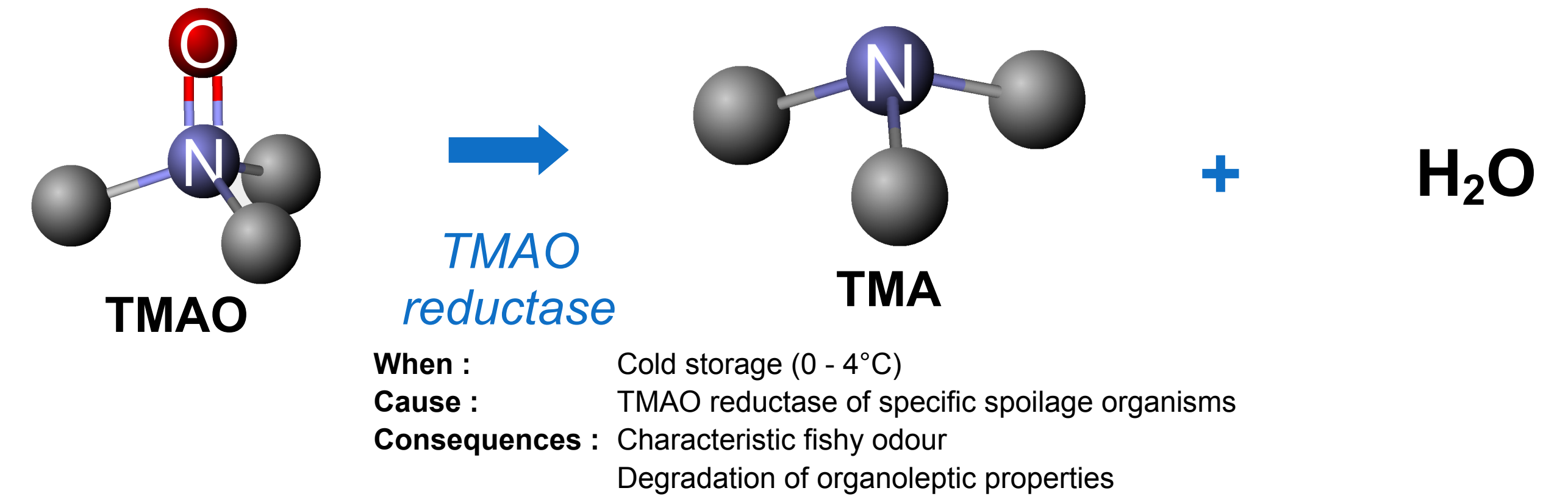


Introduction

→ 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.

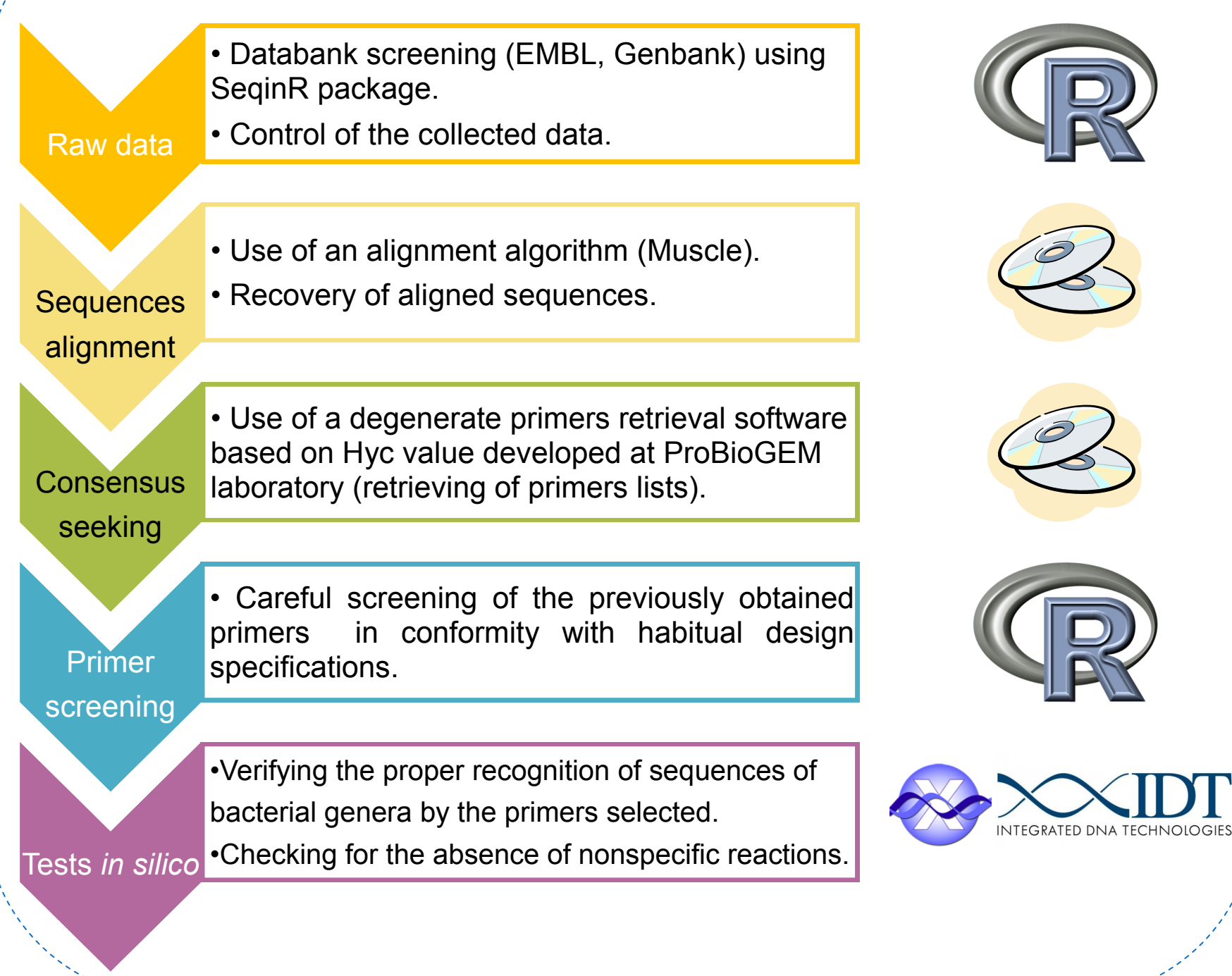
→ The major cause of the freshness decay is due to the development of spoilage flora called specific spoilage organisms (SSO). During fish spoilage SSO reduce trimethylamine N-oxide (TMAO), present in the fish flesh, thanks to an enzyme called TMAO reductase encoded by *torA* gene. This reaction leads 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 ^a	[Primers] 1 µM		[Primers] 0.5 µM	
		62°C	64°C	62°C	64°C
<i>P. phosphoreum</i> DSM2167	Intercept ^b	-3.512	-3.539	-3.679	-3.834
	R ²	0.998	0.998	0.999	0.998
	Efficiency (%) ^b	92.6	91.7	87.0	82.3
<i>P. damselae</i> CIP100540	Intercept ^b	-3.542	-3.631	-3.615	-3.813
	R ²	0.998	0.999	0.998	0.996
	Efficiency (%) ^b	91.6	88.5	89.1	82.9
<i>V. vulnificus</i> CIP 75.4	Intercept ^b	-3.512	-3.774	-3.802	-4.030
	R ²	0.993	0.999	0.999	0.994
	Efficiency (%) ^b	92.6	84.1	83.3	77.4
<i>V. alginolyticus</i> CIP 101888	Intercept ^b	-3.503	-3.810	-3.738	-3.979
	R ²	0.996	0.999	0.999	0.995
	Efficiency (%) ^b	93.0	83.0	85.1	78.4

^a Values were obtained from four replicates on 7 log DNA dilutions ranging from 15 µg mL⁻¹ to 15 pg mL⁻¹ of template DNA in the tube. ^b Efficiency was calculated according to the formula given as follows: Efficiency = 10^(-1/slope) - 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 ^a	Band at the expected size ^b	Aspecific bands	Sequencing results ^c
<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> 44LSABSM1SHW	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

^a Mean of Cq values recorded for duplicate measures, for template DNA at 15 µg mL⁻¹. ^b Existence of a band close to the expected size of 420 bp. ^c 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.

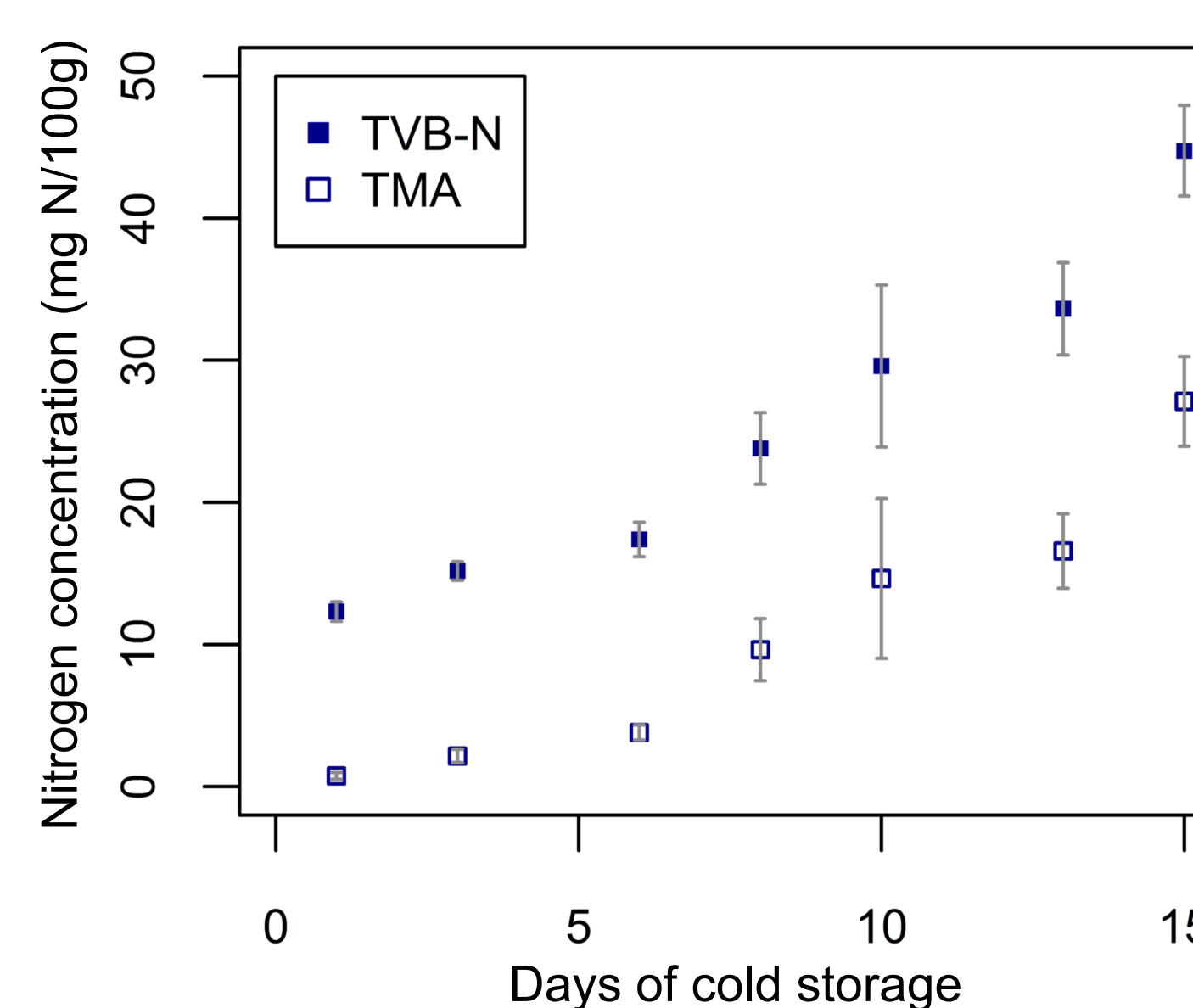


Figure 1: Average TVB-N & TMA values (n=6) per day throughout the shelf-life.

→ 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.

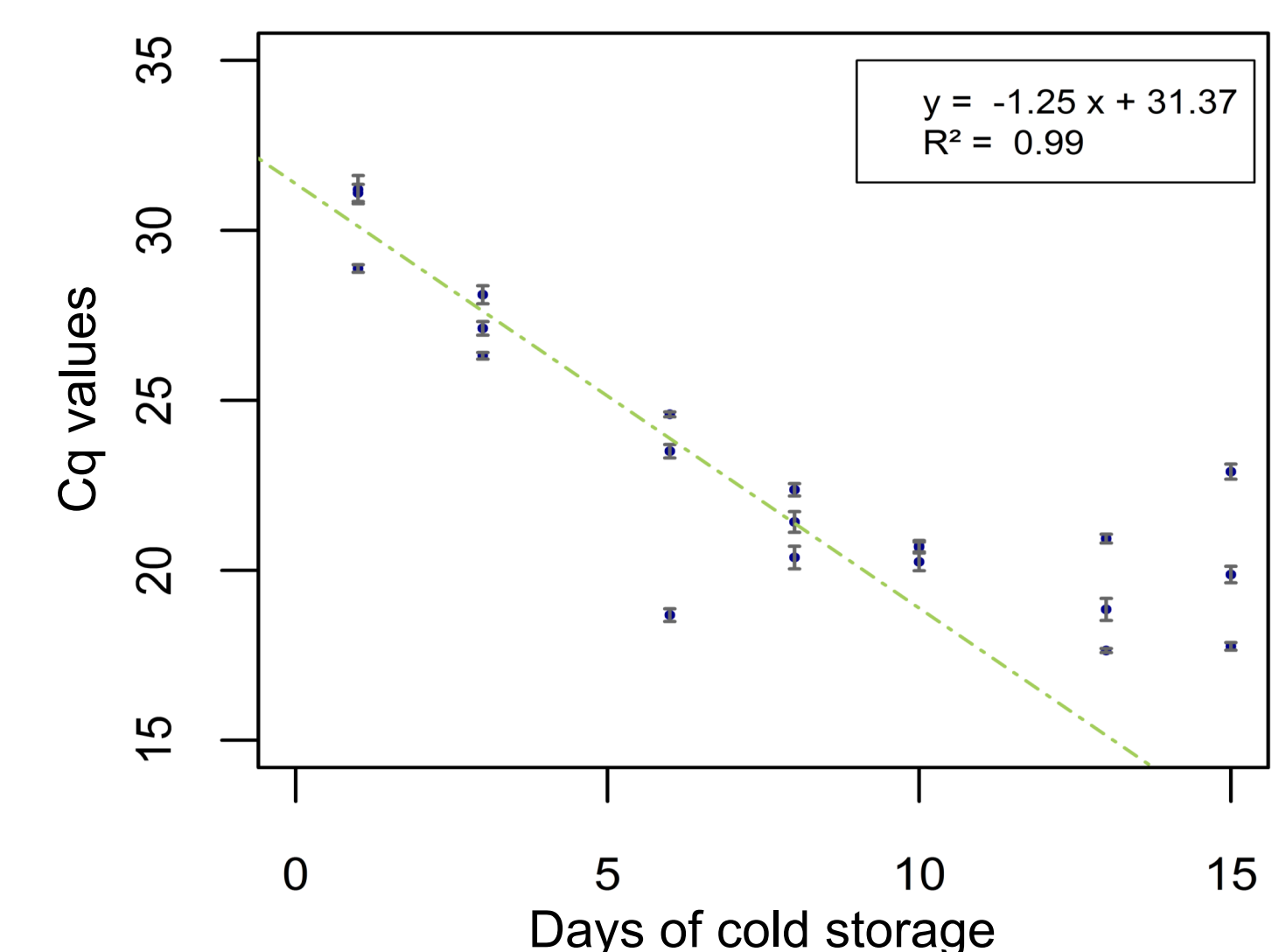


Figure 2: Mean Cq values (n=4) calculated per fish throughout the study. Green dashed line corresponds to the linearity zone from day 1 to day 8.

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

- . Clear decrease of Cq value, linear (R² = 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