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

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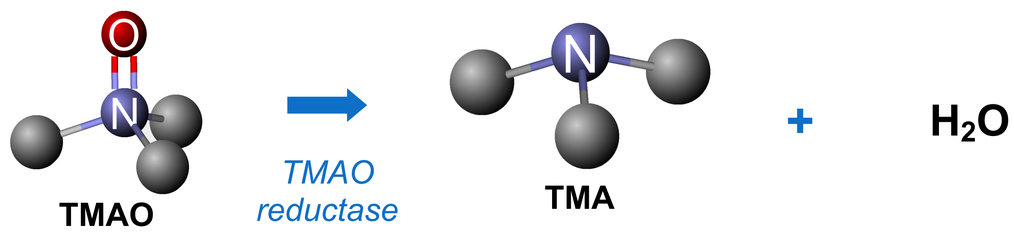
Introduction

➔ 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.



When : Cold storage (0 - 4°C)
Cause : TMAO reductase of specific spoilage organisms
Consequences : Characteristic fishy odour
Degradation of organoleptic properties

Materials & Methods

➔ Primers design and *in silico* characterization

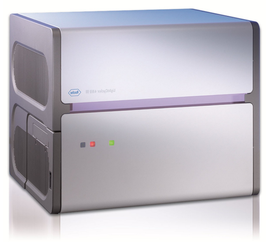
- **Raw data**
  - Databank screening (EMBL, Genbank) using SeqinR package.
  - Control of the collected data.
- **Sequences alignment**
  - Use of an alignment algorithm (Muscle).
  - Recovery of aligned sequences.
- **Consensus seeking**
  - Use of a degenerate primers retrieval software based on Hyc value developed at ProBioGEM laboratory (retrieving of primers lists).
- **Primer screening**
  - Careful screening of the previously obtained primers in conformity with habitual design specifications.
- **Tests *in silico***
  - Verifying the proper recognition of sequences of bacterial genera by the primers selected.
  - Checking for the absence of nonspecific reactions.

➔ 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 +1.0ºC 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. AmplifX 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

		[Primers] 1 µM		[Primers] 0.5 µM	
		62°C	64°C	62°C	64°C
P.phosphoreum DSM2167	Slope [a]	-3,512	-3,539	-3,679	-3,834
	Intercept [a]	37,3	38,4	39,1	41,4
	R² [a]	0,998	0,998	0,999	0,998
	Efficiency (%) [b]	92,6	91,7	87,0	82,3
P.damselae CIP100540	Slope [a]	-3,542	-3,631	-3,615	-3,813
	Intercept [a]	37,8	38,8	38,6	40,0
	R² [a]	0,998	0,999	0,996	0,996
	Efficiency (%) [b]	91,6	88,5	89,1	82,9
V.vulnificus CIP 75.4	Slope [a]	-3,512	-3,774	-3,802	-4,030
	Intercept [a]	37,1	38,5	39,2	41,2
	R² [a]	0,993	0,999	0,999	0,994
	Efficiency (%) [b]	92,6	84,1	83,3	77,1
V.alginolyticus CIP 101888	Slope [a]	-3,503	-3,810	-3,738	-3,979
	Intercept [a]	36,7	38,5	38,1	40,1
	R² [a]	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 the formula given as follow Efficiency = 10 $^{-(1/slope)}$.

- 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]
V. cholerae CIP 106974	10.98 ± 0.02	Yes	No	100 % identity with *V. cholerae* IEC224 *torA* gene
V. parahaemolyticus CNRVC 010089	11.77 ± 0.03	Yes	No	99% identity with *V. parahaemolyticus* VP2007-007 *torA* gene
V. mimicus CIP 101888	10.93 ± 0.01	Yes	No	99% identity with *V. mimicus* VM573 *torA* gene
V. fluvialis CIP 103355	13.98 ± 0.01	Yes	No	96 % identity with *V. furnissii* NCTC 11218 *torA* gene
V. metschnikovii CIP 69.14	34.22 ± 0.05	Yes	Yes	No sequencing
V. harveyi CIP 103192	11.94 ± 0.01	Yes	No	99% identity with *V. harveyi* ATCC BAA-1116 *torA* gene
V. hollisae CIP 104354	29.55 ± 0.06	Yes	Yes	No sequencing
V. furnisii CIP 102972	12.92 ± 0.01	Yes	No	98 % identity with *V. furnissii* NCTC 11218 *torA* gene
V. nigripulchritido CIP 103195	11.44 ± 0.01	Yes	No	88 % identity with *V. cholerae* IEC224 *torA* gene
S. putrefaciens DSM 6067	36.52 ± 0.11	No	Yes	No sequencing
S. baltica 14LSABSM1SHW	30.89 ± 0.01	Yes	Yes	90 % identity with *S. baltica* BA 175 *torA* gene
B. subtilis ATCC 6633	34.18 ± 0.53	Yes	No	75% identity with *V. vulnificus* CMCP6 *torA* gene
S. aureus ATCC 25923	37.54 ± 0.73	No	No	No sequencing
S. epidermidis ATCC 12228	36.65 ± 0.93	No	No	No sequencing
E. coli ATCC 25922	29.68 ± 0.15	No	Yes	No sequencing
S. enterica ATCC 13076	34.91 ± 1.00	Yes	Yes	98 % identity with *S.enterica* CT18 hypothetical protein
C. maltaromaticum	37.83 ± 0.36	No	No	No sequencing
C. divergens	34.92 ± 0.25	Yes	No	96 % identity with *V. vulnificus* CMCP6 *torA* gene
C. gilleni	31.16 ± 0.20	Yes	Yes	93 % identity with *S. baltica* BA175 *torA* gene
S. proteomaculans	32.82 ± 0.01	No	Yes	No sequencing
P. agglomerans	17.84 ± 0.22	No	Yes	No sequencing
P. fluorescens	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. baltica*.

➔ 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.

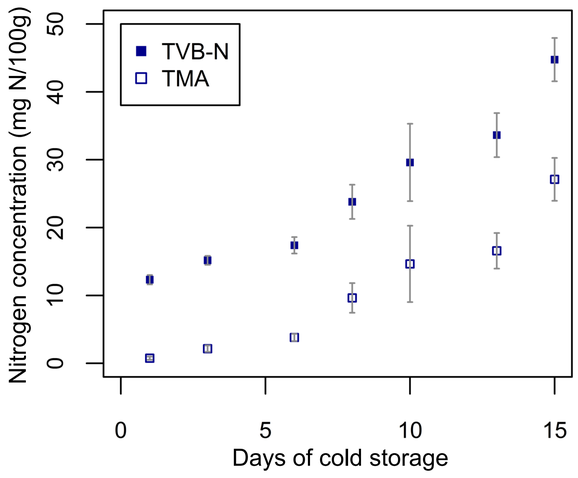


Figure 1: Average TVB-N & TMA values (n=6) per day throughout the shelf-life.

➔ 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.

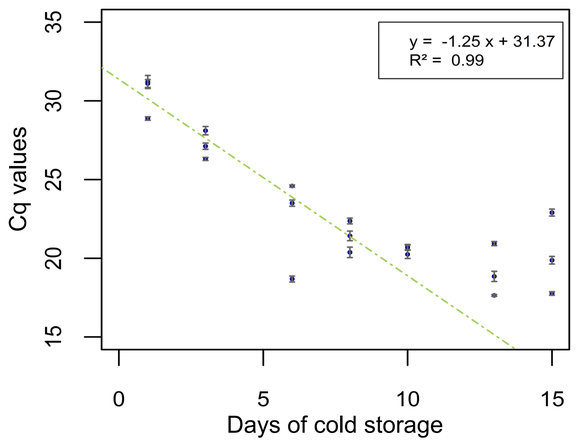


Figure 2: Mean Cq values (n=4) calculated per fish throughout the study. Green dashed line corresponds to the linearity zone from day 1 to day 8.

➔ 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).

Perspectives

➔ 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, environment 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.

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