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Implementation of methodologies for *Vibrio* spp. detection in microalgae production

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Abstract

Considering the increasing microalgae production, the main goal of this dissertation was to implement methods to detect *Vibrio cholerae*, *V. parahaemolyticus,* and *V. vulnificus* in microalgae biomass. To satisfy the customers' request for a standard method, the International Organization for Standardization (ISO) 21872-1:2017 was chosen. The final goal was to infer if it was profitable to implement the method at A4F's laboratory rather than asking a reference laboratory. A positive result for *Vibrio* spp. was never previously obtained and so its absence was expected in the biomass. The aim was to understand the number of steps required to rule out false positives on samples previously tested as negative by the reference laboratory. The method comprised four main steps: selective enrichment in Alkaline Saline Peptone Water (ASPW), isolation and identification in Thiosulfate Citrate Bile and Sucrose (TCBS) agar and CHROMagar™ and, lastly, the confirmation steps. The selective enrichment step followed by plating on CHROMagar™ and TCBS were not as selective as stated by manufactures and allowed the growth of a wide range of false positive colonies with 1 mm or less. Since *V. cholerae* positive control presented smaller colonies on TCBS, the size of colonies was first neglected as a discriminatory factor. Although some evidences indicated that these colonies were not *Vibrio* spp., further confirmation was required. The confirmation step started with Gram staining, oxidase test and examination of motility. The inability of these tests to rule out all false positives led to the non-inclusion of these steps and advancing directly to PCR. The conclusion of all the analysis was that "Potentially enteropathogenic *V. cholerae, V. parahaemolyticus* and *V. vulnificus* were not detected in 25 g of the paste according to the ISO 21872-1:2017". Additionally to the official method, the contaminants colonies did not belong to the genus *Vibrio* but that genus was detected by PCR in the secondary enrichment samples, which showed some limitations of the platting method. Even though, since not all bacteria belonging to the genus *Vibrio* are pathogenic, it did not mean, for itself, a threat to the human health. Lastly, spiked samples with the three *Vibrio* spp. were tested. Even though a high number of false positive colonies was present, the difference in size along with the complementarity of both media allowed to detect the three *Vibrio* spp. and to rule out false positives, including the colonies with less than 1 mm. The comparison between direct PCR to the enrichment broths and the official method proved that no method is ideal and PCR screening of enrichment broths was not, for itself, a reliable method to replace the ISO method. Ultimately, the financial analysis stated that the cost per analysis was approximately three times higher than the price provided by the reference laboratory. Hence, it was not cost-effective to implement the method.

Additionally, Fluorescence *In Situ* Hybridization (FISH) probes provided by Biomode SA were tested as a method to detect *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* directly in microalgae culture samples. The detection of *Vibrio* spp. would lead to the disposal of these cultures in order to avoid further costs in cultures that could not be commercialized. To counteract the autofluorescence of chlorophyll in the microalgae, Sudan Black B was successfully tested. The limit of detection obtained, 10^6 CFU mL^{-1}, was within the value expected for microscopic observation. Although, the requirement of an enrichment step, together with the prerequisite of the additional steps to inhibit the autofluorescence, turned the method time-consuming and with a detection limit above the expected values for *Vibrio* spp. in the cultures.

Keywords: *Vibrio*, ISO/TS 21872:2007, ISO 21872-1:2017, FISH, microalgae.

Resumo

As microalgas apresentam capacidade de produzir uma variedade de compostos de alto valor económico. Contudo, a contaminação das culturas com bactérias representa um problema crítico na produção à escala industrial, uma vez que a presença de organismos potencialmente patogénicos pode impossibilitar a entrada dos produtos no mercado. De forma a estabelecer a qualidade da biomassa produzida, é de fundamental importância a implementação de métodos de deteção de possíveis contaminantes. O facto da bactéria vibrio se encontrar amplamente difundida em ambientes aquáticos torna-a num possível contaminante das culturas de microalgas. Devido à sua capacidade de infetar não só animais mas também seres humanos, as contaminações representam uma ameaça preocupante para a saúde humana. *Vibrio cholerae*, *V. parahaemolyticus* e *V. vulnificus* representam as espécies detetadas com maior frequência e responsáveis por um aumento mundial de infeções transmitidas por alimentos marinhos. A sua ausência na biomassa de microalgas produzida para alimentação e rações é crucial e, consequentemente, a sua deteção deve basear-se em métodos eficazes. Como consequência do aumento da produção de microalgas, o objetivo principal desta dissertação foi implementar métodos internos para detetar estas três espécies de *Vibrio* na biomassa de microalgas. O principal método testado consistiu na ISO (International Organization for Standardization) 21872-1: 2017, baseado na presença/ausência da bactéria, de forma a satisfazer a exigência dos clientes por um método oficial. O objetivo final foi inferir se compensa implementar as análises no laboratório da A4F ou se seria preferível solicitar a sua realização a um laboratório externo de referência.

Primeiramente, foram implementadas as medidas de biossegurança requeridas para trabalhar com microrganismos de risco II no laboratório da A4F. As duas partes da ISO/TS 21872-1:2007 foram compiladas num único documento, a ISO 21872-1:2017, dedicada à deteção de *V. cholerae*, *V. parahaemolyticus* e *V. vulnificus*. Atendendo ao facto de todas as análises previamente realizadas pelo laboratório externo terem fornecido um resultado negativo para a deteção das espécies de *Vibrio,* era expectável a sua ausência na biomassa. Consequentemente, o objetivo era inferir o número de etapas necessárias para descartar falsos positivos. Para tal, as amostras analisadas foram previamente testadas pelo laboratório de referência como negativas. O método da ISO 21872-1:2017 compreendeu quatro etapas principais: enriquecimento seletivo, isolamento, identificação e confirmação. Os dois enriquecimentos seletivos sucessivos foram realizados em água peptonada salina alcalina (ASPW) e as posteriores etapas de isolamento e identificação consistiram no plaqueamento em TCBS e num segundo meio seletivo, CHROMagar™.

Em relação aos controlos positivos, as colónias obtidas em CHROMagar™ corresponderam ao esperado. Em TCBS, tirando o facto de as colónias de *V. cholerae* serem menores do que era descrito, *V. cholerae* e *V. parahaemolyticus* originaram colónias típicas. Contrariamente, as colónias de *V. vulnificus* apresentaram uma coloração amarela em detrimento de verde. Posteriormente, todos os controlos positivos foram confirmados por PCR e esta discrepância justificada pelo facto de algumas estirpes de *V. vulnificus* fermentarem sacarose, resultando na coloração amarela. Conclui-se assim que a fermentação de sacarose não representa um factor discriminatório. Não obstante, visto que o objetivo era detetar todas as espécies abrangidas pela ISO 21872-1:2017, o método não foi influenciado.

Na análise das amostras, o enriquecimento seletivo seguido de plaqueamento em CHROMagar™ e TCBS demostrou menos seletividade do que declarado pelos fabricantes, considerando que permitiu o desenvolvimento de uma ampla gama de colónias de falsos positivos com dimensões iguais ou inferiores a 1 mm. Visto que o controlo positivo de *V. cholerae* apresentou colónias menores do que o expectável em TCBS, o tamanho das colónias típicas foi negligenciado como fator discriminatório e todas as colónias verdes e amarelas em TCBS e roxas ou azuis em CHROMagar™ prosseguiram para as etapas de confirmação, resultando num método demorado. Contudo, verificou-se realmente uma discrepância entre o tamanho das colónias da amostra (1 mm ou inferior) e o tamanho esperado (2 - 3 mm) em TCBS e apenas foram obtidos aglomerados roxos ou azuis em CHROMagar™ sem colónias isoladas. Essas evidências, aliadas ao facto de que *V. cholerae* apresentou colónias típicas em CHROMagar™, poderiam indicar que essas colónias não eram as espécies de *Vibrio* em teste. Contudo, era necessário confirmação adicional. A etapa de confirmação consistiu nos testes de coloração de Gram, teste de oxidase e ausência/presença de células móveis. O género *Vibrio* corresponde a bactérias Gram-negativas, oxidase positivas e com células móveis. Contudo, nem todos os controlos positivos apresentaram células móveis e, consequentemente, este teste tornou-se inviável. Adicionalmente, a incapacidade destes testes de eliminar todos os falsos positivos levou à omissão desta etapa, prosseguindo diretamente para PCR. A conclusão de todas as análises foi "Ausência de *V. cholerae, V. parahaemolyticus* e *V. vulnificus*, potencialmente enteropatogénicos, em 25 g da pasta de acordo com a ISO 21872-1: 2017", coincidindo com os resultados fornecidos pelo laboratório externo de referência.

Adicionalmente ao método oficial, o primer para o género *Vibrio* permitiu inferir que os contaminantes que originaram as colónias de falsos positivos não pertenciam a este género. Além disso, apesar do PCR realizado diretamente no enriquecimento secundário não ter detetado nenhuma das três espécies, revelou a presença do género *Vibrio*, demostrando limitações do método microbiológico. Contudo, considerando que nem todas as bactérias pertencentes ao género *Vibrio* são potencialmente patogénicas, isto não significava, obrigatoriamente, uma ameaça para a saúde humana.

Com o objetivo de inferir se o elevado número de falsos positivos podia originar resultados falsos negativos para deteção de vibrio, a última etapa laboratorial consistiu em testar amostras artificialmente contaminadas com 10 Unidades Formadoras de Colónias (UFC) e 100 UFC de cada espécie de *Vibrio*. Em ambos os meios, foram identificadas colónias típicas, com as dimensões expectáveis. Consequentemente, embora *V. cholera* tenha originado colónias menores em TCBS, esta espécie formou colónias típicas no segundo meio seletivo. O facto de *V. cholerae* ter originado colónias típicas em CHROMagar™ confirmou a sua maior eficácia e a importância da complementaridade entre os dois meios. Concluindo, embora haja um elevado número de colónias de falsos positivos, a diferença de tamanho aliada à complementaridade dos meios permitiu detetar as três espécies de *Vibrio* e descartar falsos positivos, incluindo as colónias com menos de 1 mm em TCBS. Quanto à comparação entre PCR realizado diretamente no segundo enriquecimento e o método oficial, apenas *V. cholerae* apresentou discrepância. Esta espécie foi detetada em CHROMagar™, mas não foi detetada no enriquecimento. Isso demostra que nenhum método é ideal e que a realização de PCR diretamente em suspensões de enriquecimento não é, por si só, um método passível de substituir o método da ISO 21872-1: 2017.

Por último, de forma a averiguar a viabilidade económica da realização das análises internamente, em detrimento da sua requisição a um laboratório de referência, realizou-se a análise financeira

do método. O custo da realização da análise no laboratório da A4F demostrou ser aproximadamente três vezes superior ao preço providenciado pelo laboratório externo. Como tal, não compensava financeiramente a implementação interna deste método. Como perspetivas futuras, é expectável o desenvolvimento e aprovação de novos métodos oficiais que poderão ser mais favoráveis economicamente.

Paralelamente, considerando os recursos disponíveis, o segundo objetivo consistiu em utilizar sondas de hibridização fluorescente *in situ* (FISH), fornecidas pela Biomode SA, como método de deteção de *V. vulnificus, V. parahaemolyticus e V. cholerae* diretamente em amostras de cultura de microalgas. Durante a otimização do protocolo, um dos problemas enfrentados foi a autofluorescência da clorofila nas microalgas. Para solucionar este problema, o Negro Sudão B foi testado, com sucesso, como tratamento de redução da autofluorescência. De modo a inferir o limite de deteção do método, foi obtida a seguinte correlação entre UFC e Densidade Ótica (DO): $UFC = (10^9) \cdot (DO) + 7 \cdot 10^8$. Em seguida, as amostras de *Nannochloropsis* foram artificialmente contaminadas com diluições seriadas de concentrações conhecidas de *V. parahaemolyticus*. O limite de deteção obtido, 10^6 CFU mL^{-1}, encontrou-se dentro do valor esperado em microscopia de fluorescência. Em todas as amostras testadas, vibrio nunca foi detetado em pastas de biomassa da A4F, possivelmente como consequência de *Nannochloropsis* apresentar capacidade de suprimir quantitativamente e qualitativamente o crescimento de algumas espécies de *Vibrio*. Deste modo, mesmo que vibrio estivesse presente, seria expectável que as UFC mL^{-1} permanecessem em valores reduzidos. Consequentemente, seria necessário uma etapa de enriquecimento, por exemplo em ASPW. Contudo, juntamente com o pré-requisito das etapas adicionais para inibição da autofluorescência das microalgas, o método demostrou ser demorado e com um limite de deteção acima dos valores esperados nas culturas. Portanto, a menos que vibrio se tornasse numa contaminação comum em culturas na A4F, não seria necessário a realização de análises de rotina. Adicionalmente, este método só poderia ser implementado como análise de rotina se as contaminações de vibrio apresentassem uma elevada carga bacteriana, considerando que teria de ser superior a 10^6 UFC mL^{-1} para ser detetado. Caso estas espécies de *Vibrio* fossem detetadas, as culturas seriam descartadas imediatamente, permitindo economizar futuros recursos em culturas que não poderiam ser comercializadas. Como perspetivas futuras, deveriam ser desenvolvidas sondas com diferentes fluorocromos, como por exemplo fluorescamina.

Palavras-chave: *Vibrio*, ISO/TS 21872:2007, ISO 21872-1:2017, FISH, microalgas.

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- **A4F -** Algafuel SA
- **ASPW** - Alkaline Saline Peptone Water
- **CAC** - Codex Alimentarius Commission
- **CFU** - Colony Forming Units
- **CT** - Cholera Toxin
- **Cy3** - Cyanine-3
- **DNA** - Deoxyribonucleic Acid
- **DPO** - Dual Priming Oligonucleotide
- **dsDNA** - Double-stranded Deoxyribonucleic Acid
- **EFSA** - European Food Safety Authority
- **EPA** - Eicosapentaenoic Acid
- **FAO** - Food and Agriculture Organization of the United Nations
- **FDA** - United States Food & Drug Administration's
- **FDA-BAM** - United States Food & Drug Administration's - Bacteriological Analytical Manual
- **FISH** - Fluorescence *In Situ* Hybridization
- **ISO** - International Organization for Standardization
- **LB** - Luria Broth
- **mCPC** - Modified Cellobiose-Polymyxin B-Colistin
- **MPN** - Most Probable Number
- **PCR** - Polymerase Chain Reaction
- **PNA** - Peptide Nucleic Acids
- **qPCR/ Real-Time PCR** - Real-time Polymerase Chain Reaction
- **RNA** - Ribonucleic Acid
- **rRNA** - Ribosomal Ribonucleic Acid
- **SDSPS** - sodium dodecyl sulfate polymyxin sucrose
- **SNA** - Saline Nutrient Agar
- **TCBS** - Thiosulfate Citrate Bile and Sucrose
- **TCP** - Toxin-Coregulated Pili
- **TDH** - Thermostable Direct Hemolysin
- **TRH** - Thermostable Direct-Related Hemolysin
- **TSAT** - Soya Peptone Triphenyl Tetrazolium Chloride
- **TSI** - Saline Triple sugar Iron
- **UV** - Ultraviolet
- **VAT** - Value Added Tax
- **VBNC** - Viable But Not Culturable
- **WHO** - World Health Organization

Chapter 1 - Introduction

1.1 Microalgae - a source of high-value compounds

Microalgae are microscopic, unicellular, colonial, or filamentous organisms that can grow photoautotrophically (Mata *et al.*, 2010; Richmond, 2004). Even if their photosynthetic mechanism is similar to higher plants, they present a higher photosynthetic efficiency, converting solar energy to biomass. This benefit is a result of its simple cellular structure, large volumetric surface and the growth in aqueous medium, which results in greater access to CO_2 and nutrients (Khan *et al.*, 2009). The extreme diversity of this heterogeneous group, comprising eukaryotic and prokaryotic microorganisms, combined with their high productivity, encompasses organisms with the capability to produce high-value compounds (**Figure 1.1**). Thus, microalgae are of economic interest (Koller *et al.*, 2014; Mata *et al.*, 2010; Richmond, 2004; Safi *et al.*, 2014).

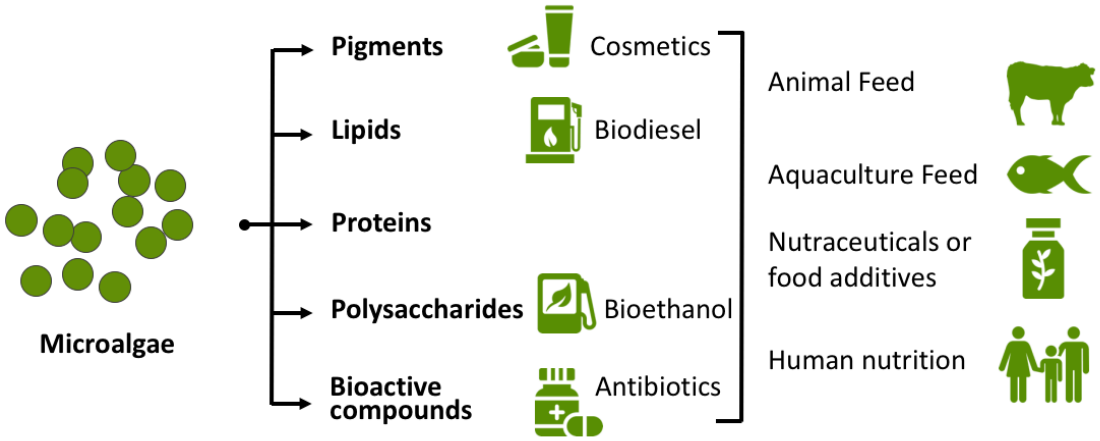


Figure 1.1 – High-value compounds produced by microalgae.

Considered as one of the most promising candidates for large scale cultivation is the microalgae *Nannochloropsis* (Safafar *et al.*, 2016). Up to date, *Nannochloropsis* genus comprises seven species, including six marine (*N. australis, N. gaditana, N. granulata, N. oceanica, N. oculata, and N. salina*) and one freshwater (*N. limnetica*) (Guiry, 2014). Species of this genus are unicellular eukaryotic microorganisms with a diameter varying from 2 to 5 μm (Wang *et al.*, 2014). They are of industrial interest due to the ability to synthesize not only high amounts of lipids for biodiesel production but also Eicosapentaenoic Acid (EPA) for nutraceuticals (Hoffmann *et al.*, 2010; Ma *et al.*, 2014). Additionally, species such as *N. gaditana* and *N. oceanica* have been used as feed in aquaculture as an alternative source of EPA from fish meal (Ferreira *et al.,* 2009; Ma *et al.*, 2016; Nakase *et al.*, 2013).

The economic potential of microalgae is undoubted, although the possible contamination of microalgae cultures with bacteria represents a major problem in industrial production. This can not only affect the yields but the presence of potentially pathogenic organisms, such as *Vibrio* spp., could also prevent the final product from entering the market (Lee *et al.*, 2019). Therefore, to establish the quality of the produced biomass it is of crucial importance to implement methodologies to detect the contaminants that can be present in microalgae production.

1.2 Vibrio bacteria

Numerous bacterial pathogens accountable for a range of clinical cases are often encountered in seawater, including *Vibrio* spp., *Escherichia coli*, *Salmonella* spp., *Proteus* spp., *Pseudomonas aeruginosa*, *Aeromonas hydrophila,* and *Streptococcus aureus* (Baker-Austin *et al.*, 2018). Regarding *Vibrio* spp., they are broadly distributed worldwide in aquatic environments, from fresh-water, estuarine to marine environments. Hence, they represent dangerous pathogens that can occur in microalgae cultures (Bhunia, 2018). Vibrios are found free-living in water, associated with inanimate surfaces or with aquatic organisms like zooplankton and phytoplankton, aquatic animals, such as seabirds, and seafood. They also present the ability to form biofilms on the abiotic and biotic surfaces, like shells, which is an important survival and colonization strategy in aquatic environments and, possibly, in microalgae photobioreactors (Baker-Austin *et al.*, 2018; Bhunia, 2018).

The European Food Safety Authority (EFSA) highlighted that rising seawater temperatures due to global warming is leading to an increased incidence of *Vibrio* spp. infections in countries around the globe (EFSA, 2016). Indeed, vibrios are of significant concern in both developed and developing countries. They comprise important pathogens for farmed animals and humans that can acquire infections after consumption of raw or undercooked contaminated seafood, polluted drinking water or exposure of skin lesions to contaminated water or animals (Baker-Austin *et al.*, 2018; Tantillo *et al.*, 2004; Thompson *et al.*, 2006). Due to its ability to infect not only marine animals but also humans, vibrio contaminations pose a serious threat to human health. This underlines the importance of its absence in microalgae biomass produced for food and feed (Austin, 2010). *Vibrio* is a risk II microorganism, a pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment (Council of the European Union, 2000).

Members of the genus *Vibrio* are curved or rod-shaped, with a size of 0.5 - 0.8 μm in width and 1.4 - 2.6 μm in length, and motile, most of them presenting a single polar flagellum. They are Gram-negative bacteria, oxidase positive (the only exception is *V. metschnikovii*), use d-glucose as the main carbon source, and are facultative anaerobes. *Vibrio* bacteria do not produce spores (asporogenous) and cell division is by an asexual process called binary fission (Bhunia, 2018; Garrity *et al.*, 2005; Tantillo *et al.*, 2004). **Table 1.1** presents differential characteristics of several *Vibrio* spp. and their association with human and animal diseases.

Table 1.1 - Differential characteristics of several *Vibrio* spp. and their association with human and animal diseases. +, - and D correspond to 100 %, 0 % and 21 % - 79 % of positive results, respectively. (+) and (-) correspond to 80 % and 20 % of positive results, respectively. Adapted from Austin (2010).

Characteristic	*V. alginolyticus*	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. metschnikovii*	*V. mimicus*	*V. parahaemolyticus*	*V. vulnificus*
Oxidase production	+	+	+	+	-	+	+	(+)
Arginine dihydrolase	–	–	+	+	D	–	–	–
Lysine decarboxylase	+	+	–	–	–	+	+	+
Gas from glucose	–	–	–	+	–	–	–	–
Indole production	+	+	D	–	–	+	+	+
Voges Proskauer reaction	(+)	+	–	–	+	–	–	–
Utilization of cellobiose	–	–	D	D	–	–	–	(+)
Growth in 0% NaCl	–	+	+	+	–	+	–	–
Growth in 0.3% NaCl	–	+	+	+	+	+	–	+
Growth in 12% NaCl	+	–	–	(–)	–	–	–	–
Cause of wound infections in humans	+		+					+
Cause of gastro-intestinal disease in humans		+	+	+	+	+	+	+
Cause of fish disease	+	+		+			+	+
Cause of invertebrate disease	+	(+)	+		+	+	+	+

The occurrence and distribution of *Vibrio* spp. in aquatic environments is affected by major factors such as water temperature and salinity, nutrient availability, and association with marine organisms. For *Vibrio* spp. in general, the optimum pH range is 8.0 - 8.8, but most species of vibrio will grow between pH 6.5 and 9.0. Most vibrios do not grow at 4 °C and in media with 0 % or 12 % (m V^{-1}) NaCl. Most of them occur when water salinity is from 0.5 % to 3 % (m V^{-1}), but they may also occur outside the optimum range in case of elevated nutrient concentrations and high water temperatures. The requirement of Na^+ for growth is widespread among most *Vibrio* spp. but *V. cholerae, V. mimicus, V. fluvialis,* and *V. furnissii* can grow in 0 % (m V^{-1}) NaCl. The distribution in freshwater, even if restricted, is dangerous due to the increment of encounters between humans and pathogenic vibrios. The optimal growth temperature range is between 20 °C and 37 °C, but they are able to grow over 40 °C. During warmer months, vibrio counts are very high, with the highest concentrations occurring when water temperature is between 20 °C and 30 °C. Additionally, at temperatures over 30 °C or below 10 °C counts noticeably reduce (Baker-Austin *et al.*, 2018; Bhunia, 2018; Percival *et al.*, 2014; Tantillo *et al.*, 2004; Thompson *et al.*, 2006).

Indeed, water temperatures on either side of the range strictly affect bacterial growth together with nutrient deficiency and salinity extremes. Under these adverse conditions, *Vibrio* spp. carry out a survival strategy based on the Viable But Not Culturable (VBNC) state, also called metabolically Active But Non-Culturable (ABNC) (Bhunia, 2018; Colwell *et al*., 1985; Jiang & Chai, 1996; Oliver *et al*., 1995; Percival *et al.*, 2014). While, in a dormant state, metabolic activity persists below the detection levels, in the VBNC state, the metabolic activity is detectable, but the bacteria cannot be cultured under standard laboratory procedures. Under appropriate conditions, bacteria can revive from this VBNC state. The trigger mechanism for resuscitation is still unidentified, but in many bacteria this appears to happen unexpectedly, either when they are present in environments, foods or during infection in the human body (Ramamurthy *et al*., 2014). For *V. parahaemolyticus*, *V. cholerae,* and *V. vulnificus*, resuscitation from the VBNC state, induced by low temperatures, occurred after the removal of the temperature stress. Indeed, resuscitation is facilitated by a gradual increase in temperature in a nutrient free medium which may be analogous to the water temperature increase, elucidating the commonly season-dependent distribution of *Vibrio* spp. (Jiang & Chai, 1996; Oliver *et al*., 1995; Ravel *et al*., 1995; Whitesides & Oliver, 1997). This problem is even more complex for potential bacterial pathogens, since microorganisms in VBNC state may remain pathogenic. Oliver (1995) suggested that cells of *V. vulnificus*, following *in vivo* resuscitation, were able to reactivate their pathogenic potential and cause disease. Other studies proved that *V. parahaemolyticus* and *V. cholerae* VBNC forms could be resuscitated and retain their virulence properties (Baffone *et al*., 2003; Colwell *et al*., 1996). For their undetectability by conventional methods of food and water analyses, VBNC pathogenic bacteria, including *Vibrio* spp., present a threat to food safety and public health (Johnston *et al*., 2002).

The genus *Vibrio,* a member of the Vibrionaceae family and Gammaproteobacteria class, contains 63 species. *V. cholerae* (serotypes O1 and O139)*, V. parahaemolyticus*, *V. vulnificus* (biotype 1, 2 and 3), *V. mimicus*, *V. fluvialis*, *V. alginolyticus*, *V. furnissii*, *V. metschnikovii*, and *V. cincinnatiensis* are among the 12 *Vibrio* spp. known as pathogenic to humans (Bhunia, 2018; Robert-Pillot *et al.*, 2014; Thompson *et al.*, 2004). *Vibrio* infections are generally divided into three well recognized clinical syndromes: gastroenteritis, primary septicemia and wound infection (Tantillo *et al.*, 2004). The association of the pathogenic *Vibrio* spp. with these clinical syndromes is presented in **Table 1.2.** *V. cholerae, V. parahaemolyticus*, *V. vulnificus*, discussed in detailed above, are the most frequently reported for human infections and responsible for a worldwide increase of seafood-borne infections (Robert-Pillot *et al.*, 2014; Thompson *et al.*, 2004).

Table 1.2 - Association of pathogenic *Vibrio* spp. with the three major clinical syndromes. Adapted from Tantillo *et al.* (2004).

Vibrio species	Primary septicaemia	Gastroenteritis	Wound infections
V. cholerae O1	*	+++	+
V. cholerae non-O1	+	+++	++
V. parahaemolyticus	+	+++	++
V. vulnificus	+++	++	+++
V. fluvialis	*	++	*
V. alginolyticus	+	*	+++
V. furnissii	*	+	*
V. mimicus	*	++	++
V. metschnikovii	+	+	*

+++, most common association; ++, possible association; +, rare association.
*, The association remains to be firmly established.

1.2.1 *Vibrio cholerae*

V. cholerae, with a size of 0.7 - 1.0 µm in width per 1.5 - 3.0 µm in length, grows at a minimum temperature of 15 °C, a maximum temperature of 45 °C and a pH range of 6 - 10. Even though *V. cholerae* does not require salt for growth, it is able to grow at concentrations up to 6 % (m V^{-1}) NaCl (Bhunia, 2018). *V. cholerae* represents one of the most important members of the *Vibrio* genus. In 1854, Pacini discovered the first *Vibrio* sp., *V. cholerae,* the causative agent of cholera, while studying outbreaks of this disease in Florence. In the same period, John Snow revealed that the cholera outbreak in London, that resulted in 616 deaths, was due to contaminated water. In 1884, Robert Koch isolated *V. cholerae* on gelatin plates. Even though the outbreaks of cholera dates back to Hippocrates times, in our days epidemic and pandemic cholera still occur with global repercussions. According to the Bulletin of the World Health Organization (WHO) (2012), *V. cholerae* infects approximately 3 to 5 million people worldwide with 100,000 to 120,000 deaths annually. It represents a severe disease mainly in developing countries as a result of poor water supplies and sanitation. Cholera is endemic in many parts of Asia and Africa. Even though autochthonous cholera has been eradicated from most areas of the developed world, imported contaminated food can represent a potential source of sporadic infections (Bhunia, 2018; Tantillo *et al.*, 2004; Thompson *et al.*, 2004).

Infection due to *V. cholerae* begins with the ingestion of contaminated water or food and the transmission is through the fecal-oral route. All strains that cause cholera carry virulence genes for Cholera Toxin (CT) and Toxin-Coregulated Pili (TCP). Using the toxin-coregulated pili, it adheres to the intestine epithelium and produces an enterotoxin, CT, which affects the ion transport pumps and junctional integrity and results in extensive fluid and ion losses, causing an intense watery diarrhea that may lead to death (Bhunia, 2018). Nevertheless, *V. cholerae* causes infections ranging from asymptomatic to a very serious watery diarrhea known as 'rice water stools'. Cholera symptoms include abrupt watery diarrhea, occasional vomiting and abdominal cramps. Loss of water and electrolytes leads to dehydration and further symptoms quickly develop (Bauerfeind *et al*., 2016; Bhunia, 2018). In cases of cholera gravis involving severe dehydration, up to 70 % of patients can die. However, when treated with rehydration therapy less than 1 % of cases are fatal (Bhunia, 2018; Public Health Agency of Canada, 2011). Infective doses of *V. cholerae* are high in healthy individuals. Hornick *et al*. (1971) showed that ingestion of 10^8 Colony Forming Units (CFU) in water can induce diarrhea in 50 % of adult volunteers and 10^{11} organisms can induce 'rice water stools'. The incubation period can range from a few hours to 5 days after infection and can last for 2 to 12 days (Bauerfeind *et al.*, 2016; Bhunia, 2018).

1.2.2 *Vibrio vulnificus*

V. vulnificus was first isolated in 1964, although it was mistakenly identified as a strain of *V. parahaemolyticus* and only in the 1970s it was recognized as a distinct species (Strom & Paranjpye, 2000). *V. vulnificus* inhabits estuarine and marine environments throughout the world. It is an obligate halophilic bacterium with an optimum salt requirement of 1 to 2 % (m V^{-1}) NaCl. This bacterium is widespread in warm waters where the temperature rounds 20 °C and has been isolated from temperatures ranging from 9 °C to 31 °C. *In vitro* growth of environmental isolates revealed that the optimum growth temperature was 37 °C (Bhunia, 2018; Kelly, 1982; Strom & Paranjpye, 2000). *V. vulnificus* is one of the main bacterial pathogens that cause disease in fish in aquaculture (Toranzo *et al.,* 2005). Even more, of all the human pathogenic vibrios, *V. vulnificus* is considered the most infectious, predominantly in immunocompromised hosts. Consumption of raw or undercooked seafood, such as oysters, is connected to outbreaks (Bhunia, 2018).

V. vulnificus infections present one of three major clinical syndromes: gastroenteritis, wound infections, and primary septicemia. Gastroenteritis symptoms are associated with abdominal pain, vomiting, and diarrhea and no fatalities from *V. vulnificus* gastroenteritis have been reported. Contrarily, the mortality rate of septicemic infection is about 50 %, and for wound infection it ranges from 20 % to 30 % in patients with other underlying health conditions (Bhunia, 2018). The most significant form of disease is septicemia, which typically follows ingestion of raw or undercooked oysters (Thompson *et al.*, 2006). From the intestinal tract, it invades epithelial cells, produces hemolysin inducing apoptosis, which facilitates the invasion of bacteria into the bloodstream. This sort of septicemia occurs mainly in immunosuppressed people (Bhunia, 2018; Thompson *et al.*, 2004). Notwithstanding, *V. vulnificus* can also be contracted as a result of preexisting cuts or acquired during recreational or occupational activities associated with the marine environment or the seafood industry. From all the infections caused by *Vibrio* spp., *V. vulnificus* is responsible for most wound infections. The infection can spread rapidly to tissue necrosis and, under extreme conditions, require amputation of limbs. To treat the infection, antibiotic therapy is required (Bhunia, 2018). The infective or lethal dose of *V. vulnificus* for humans is still unknown but is estimated to be as low as 100 CFU. The average incubation period of the disease is 26 h, but in the case of wound infection, the incubation period is much shorter, about 16 h (Bhunia, 2018; Strom & Paranjpye, 2000).

1.2.3 *Vibrio parahaemolyticus*

V. parahaemolyticus was first discovered in the 1950s by a Japanese scientist, when the organism caused an outbreak affecting 272 people, resulting in 20 deaths due to the consumption of fish (Fujino *et al.*, 1953). *V. parahaemolyticus* is distributed in the marine environment (estuarine) and grows within a temperature range of 15 - 44 °C, with optimal growth temperatures between 35 °C and 39 °C. It is a moderate halophile, requiring salt for survival, and is capable of growth at 1 - 9 % (m V^{-1}) NaCl (Bhunia, 2018; Yeung & Boor, 2004). This bacterium is the leading cause of seafood-borne bacterial gastroenteritis in the world. Often associated with the consumption of contaminated raw or undercooked seafood, it is accountable for 20 % to 30 % of food poisoning cases associated with seafood (Bhunia, 2018; Raghunath, 2015).

V. parahaemolyticus infections usually present one of three major clinical syndromes: gastroenteritis, wound infections, and septicemia. Wound infection and sepsis can result from contact with the bacteria. Fatal cases of septicemia may occur in immunocompromised patients or with a pre-existing medical condition (Yeung & Boor, 2004). The most common *V. parahaemolyticus* infection is gastroenteritis.

Symptoms include diarrhea (watery or bloody) and infection is usually self-limiting and can be treated with oral rehydration alone (Nair *et al*., 2007). Infectious dose ranges between ingestion of 10^5 and 10^7 CFU. Incubation period is usually 15 h after infection, with a range of 4 - 96 h and disease may last for 2 - 3 days (Bhunia, 2018; Nair *et al*., 2007, Yeung & Boor, 2004). Though the detailed mechanism of pathogenesis causing fluid loss resulting in diarrhea is still unclear, the hemolysins, Thermostable Direct Hemolysin (TDH) and TDH-Related Hemolysin (TRH), have been considered important in causing gastroenteritis. Raimondi *et al.* (2000) proposed that TDH performs as a porin in the intestinal absorptive cells and allows the influx of various ions. A high concentration of TDH increases the number of porin channels, results in ionic influx, ending in cell swelling and apoptosis (Bhunia, 2018; Raimondi *et al.*, 2000). Only some strains of *V. parahaemolyticus* are pathogenic, being typically associated with the production of TDH and TRH. However, about 10 % of clinical strains do not contain *tdh* and/or *trh*. The fact is that more than 90 % of clinical *V. parahaemolyticus* isolates, but less than 1 % of food or environmental strains, contain these genes. Nevertheless, isolates lacking *tdh* and/or *trh* remain pathogenic, indicating other virulence factors exist such as extracellular proteases, biofilm, and siderophore, and may be highly cytotoxic to human gastrointestinal cells (Blanco-Abad *et al.*, 2009; Raghunath, 2015).

1.3 General methods to detect *Vibrio* spp.

As previously mentioned, *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus* are the most frequently reported and responsible for a worldwide increase of seafood-borne infections (Robert-Pillot *et al.*, 2014). Therefore, early detection of these pathogenic *Vibrio* spp. is crucial and should rely on effective methods, while avoiding misleading results.

In general, conventional culture based methods involve a selective pre-enrichment of samples, plating onto selective agar media followed by morphological, biochemical and serological characterization (Tantillo *et al.*, 2004). However, these methods present limitations, detecting only culturable bacteria, ignoring the bacteria that cannot grow on media but are capable of causing disease, remaining a significant public health threat (Colwell *et al*., 1996). Furthermore, the traditional phenotypic assays present low sensitivity and may fail to detect strains of bacteria at low concentrations in the samples or with uncommon phenotypes (Aono *et al*., 1997; Kaysner *et al*., 1994). Moreover, traditional techniques are burdensome and often require 5 to 10 days to be performed, or even more (Candrian, 1995; Tantillo *et al.*, 2004). As a consequence, over the past 20 years, the identification of *Vibrio* spp. has been improved by the development of molecular techniques (Bonnin-Jusserand, 2019), briefly reviewed bellow. The molecular approach delivers faster, more sensitive, and specific tools, that also allow the detection of low numbers of bacteria, VBNC microorganisms that are difficult to identify by traditional means (Tantillo *et al.*, 2004).

Some molecular methods for *Vibrio* spp. detection, including their pathogenicity, consist on Fluorescent *In Situ* Hybridization (FISH), Polymerase Chain Reaction (PCR), multiplex PCR to detect various *Vibrio* spp. and pathogenic traits (Jeyasekaran *et al.*, 2011; Kim *et al*., 2015; No *et al.*, 2011; Schauer *et al*., 2012). Real-time polymerase chain reaction (Real-Time PCR or qPCR) has also been applied for *Vibrio* spp., including sequential multiplex to distinguish between five *Vibrio* spp. in fish and seafood, viable and dead bacteria and to determine *V. cholerae* serogroup (Espiñeira *et al.,* 2010). Several qPCR methods have been developed with the aim of targeting either single species (Baker-Austin *et al.*, 2012; Robert-Pillot *et al.,* 2010) or a multiplex approach to detect several *Vibrio* spp. in a single run (Kim & Lee 2014; Messelhäusser *et al.,* 2010; Robert-Pillot *et al.,* 2014). Garrido-Maestu *et al.* (2014) established a multiplex qPCR method to detect *V. cholerae*, *V. parahaemolyticus,* and

V. vulnificus, presenting a higher number of positive results than with the International Organization for Standardization (ISO) standard method. Regarding PCR derived methods, Xu *et al.* (2017) developed a Dual Priming Oligonucleotide (DPO) multiplex PCR assay, singular in terms of primer design, to detect *V. cholerae*, *V. parahaemolyticus,* and *V. vulnificus* in a single run. Tested with seafood samples after an enrichment period, this method obtained higher specificity than PCR. Further review of the two molecular methods applied in this dissertation, FISH and qualitative polymerase chain reaction (PCR), is presented below.

1.3.1 FISH

FISH is a molecular technique which applications are too numerous to list completely, including phylogenetic, ecologic, diagnostic, comprising food control, and environmental studies in microbiology (Amann & Fuchs, 2008; Bishop, 2010; Moter & Göbel, 2000; Rohde *et al*., 2015; Wagner *et al*., 2003). It can be used to identify prokaryotic cells without cultivation, representing a rapid and highly specific technique for whole-cell detection. FISH detects nucleic acid sequences through fluorescently labeled nucleic acid probes that hybridize specifically to the complementary target sequence within the intact cell (Moter & Göbel, 2000; Rohde *et al*., 2015).

The earliest *in situ* hybridization (ISH), performed in the 1960s, was not fluorescent, instead the probes were labeled with radioisotopes (Gall & Pardue, 1969). However, several drawbacks of isotopic hybridization led to the development of new techniques (Levsky & Singer, 2003). The first application of fluorescence *in situ* detection came in 1980, with a probe for a specific deoxyribonucleic acid (DNA) sequence labeled with a fluorophore directly to the 3' end of Ribonucleic Acid (RNA) (Bauman *et al.*, 1980). Giovannoni *et al.* (1988) introduced it into bacteriology, applying radioactively labeled oligonucleotide probes targeting Ribosomal Ribonucleic Acid (rRNA) for the microscopic detection of bacteria. Furthermore, in 1989, DeLong *et. al.* achieved the detection of single microbial cells with the first fluorescently labeled oligodeoxynucleotides complementary to 16S rRNA. After this development, radioactive probes were progressively replaced with fluorescently labeled probes. Compared to the radioactive probes, fluorescent probes allowed significant improvements in resolution, cost, safety, and speed, not requiring additional detection steps. Furthermore, since fluorescent probes can be labeled with dyes of different emission wavelengths, it enables the simultaneous detection of multiple targets, for example, numerous contaminant microorganisms detection in microalgae reactors (Levsky & Singer, 2003; Moter & Göbel, 2000).

1.3.1.1 Protocol

Standard FISH protocols involve five main steps (**Figure 1.2**): fixation and permeabilization of the sample, hybridization of the fluorescent probe, washing away unbound probes and detection of labeled cells by fluorescence microscopy or flow cytometry (Cerqueira *et al*., 2008; Moter & Göbel, 2000).

Fixation and permeabilization occur concurrently. These steps aim to prevent cell lysis, to preserve cellular components and morphology and to protect the RNA from degradation by endogenous RNAses while intending to increase the cell wall permeability, subsequently allowing the entrance of the fluorescent probe. These steps include the application of precipitating agents like ethanol, cross-linking agents such as paraformaldehyde or a mixture of both (Moter & Göbel, 2000; Paavilainen *et al.*, 2010; Wagner *et al.*, 2003). The hybridization step must be carried out under stringent conditions for proper annealing of the probe to the target sequence when complementary sequences are present. The washing step guarantees that all loosely bound and unbound labeled probes are removed from the sample,

providing specificity to the method (Amann & Fuchs, 2008; Moter & Göbel, 2000; Silverman & Kool, 2007; Stender *et al.,* 2002). The last step, detection of labeled cells, can be carried out by flow cytometry or visualization on fluorescence microscopy (Amann & Fuchs, 2008). Additionally to the general protocol, in food microbiology, other steps could be required such as sample preparation, homogenization, and pre-enrichment procedures (Rohde *et al*., 2015).

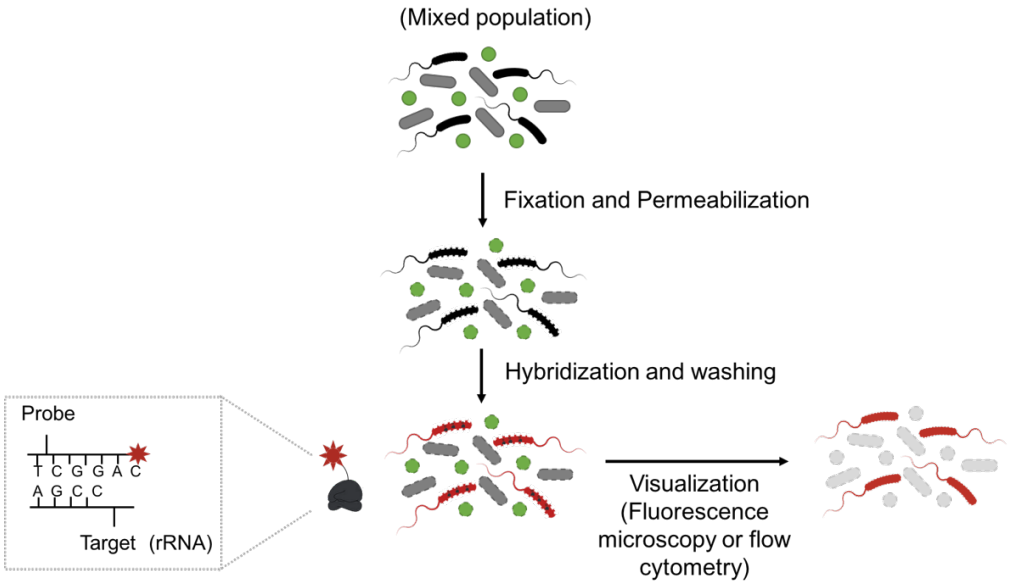


Figure 1.2 - Scheme of FISH method. The procedure includes the following steps: fixation and permeabilization, hybridization of probes and washing. Lastly, visualization is by fluorescence microscopy or flow cytometry.

In most cases, the probes used for FISH identification of Bacteria or Archaea target a sequence of the 16S rRNA and for Eukaryotes a sequence of the 18S rRNA. The rRNA choice as a target molecule is due to its high abundance, consequently resulting in high fluorescence intensities. Even more, rRNA is frequently used as a phylogenetic marker due to its genetic stability and structure with conserved and variable regions (Silverman & Kool, 2007; Stender *et al.*, 2002; Woese, 1987). When selecting a FISH probe, it is crucial to take into account its ability to penetrate the outer cell layer, specificity, and sensitivity. Specificity is calculated as the percentage of the number of strains of the microorganism of interest detected by the probe, divided by the total number of microbial strains detected by the probe (Guimarães *et al.,* 2007). Sensitivity is expressed as a percentage, where the number of strains of the microorganism of interest detected by the probe is divided by the total number of strains of the microorganism of interest present in the database (Almeida *et al*., 2010; Guimarães *et al*., 2007).

1.3.1.2 Drawbacks

Based on Moter and Göbel review (2000), FISH presents some possible drawbacks. False positive results can be obtained due to autofluorescence of microorganisms or lack of specificity. False positive results due to autofluorescence represent one of the most striking problems in FISH. This matter has been described for certain bacteria of the genus *Pseudomonas* (Brown & Lowbury, 1965), *Legionella* (Wilkinson *et al.,* 1990), *Rhodospirillum centenum* (Albrecht-Buehler, 1997), for cyanobacteria (Schönhuber *et al.,* 1999), for a variety of fungi (Graham, 1983) and archaea (Sørensen *et al.,* 1997). Furthermore, other components in cells present natural fluorescence activity such as chlorophyll in plant material, flavins, porphyrins and lipofuscin which are typically in mitochondria and lysosomes in numerous cell types. Some extra-cellular matrix components such as collagen and elastin, and some exogenous dyes also present autofluorescence (Oliveira *et al.,* 2010).

To reduce autofluorescence, different procedures could be used such as irradiation with visible or long-wavelength ultraviolet (UV) light and photobleaching, a process in which fluorescent molecules lose their ability to fluoresce due to cleavage of covalent bonds caused by light (Neumann & Gabel, 2002; Song *et al.*, 1995). Another procedure is using Sudan Black B, frequently mentioned in the literature, mainly known for quenching the autofluorescence of lipids and some lipoproteins (Heaney *et al*., 2011; Neumann & Gabel, 2002; Oliveira *et al.,* 2010; Schnell *et al.,* 1999). Nonetheless, Sudan Black B is not specific to lipids and therefore, can be used for other constituents (Schnell *et al.*, 1999).

Additionally, false negative results can be a result of insufficient probe penetration which depends on the structure of the bacterial cell wall. Another explanation is that not all sequences are equally accessible for probes as a result of higher order structures of the target such as the three-dimensional structure of the rRNA (Moter & Göbel, 2000). Also, low rRNA content can cause false positives since, although rRNA content is usually highly abundant, it may vary significantly, not only between species but also within cells of the same strain according to their physiologic state which is directly correlated with the growth rate. Low physiological activity can result in low signal intensity and can result in false negative results (DeLong *et al*., 1989; Kemp *et al*., 1993; Poulsen *et al*., 1993; Wallner *et al*., 1993). Additionally, the low affinity between the probe and the target has been reported as the cause of why DNA probes fail to give intense signals (Yilmaz *et al*., 2006). These problems associated with DNA probes led to the development of improved probes in the form of synthetic nucleic acid analogs named DNA mimics. Since then, Peptide Nucleic Acids (PNA) and other nucleic acid analogues were created (Cummins *et al*., 1995; Egholm *et al*., 1993; Koshkin *et al*., 1998; Nielsen *et al*., 1991).

1.3.1.3 PNA probes

PNA molecules are DNA mimics with the negatively charged sugar-phosphate backbone of DNA replaced by a neutral polyamide backbone composed of repetitive units of N-(2-aminoethyl) glycine (**Figure 1.3)** (Nielsen *et al.*, 1994). Individual nucleotide bases are attached to each of the units enabling PNA to hybridize with complementary nucleic acid targets obeying the Watson and Crick base-pairing rules. The synthetic backbone provides PNA probes with unique hybridization characteristics, such as faster and stronger binding to complementary targets, in part because of the absence of electrostatic repulsion (Egholm *et al*., 1993).

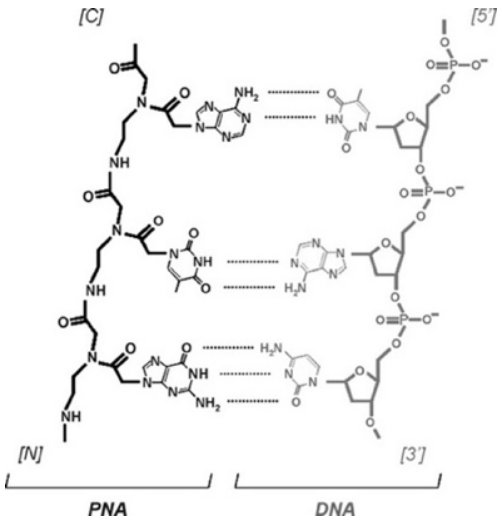


Figure 1.3 - Schematic chemical representation of a DNA and PNA molecule. PNA hybridizes in antiparallel orientation with complementary DNA. Dotted lines represent hydrogen bonding between complementary nucleobases. Adapted from Mateo-Martí and Pradier (2010).

PNA probes, being shorter than DNA probes, can easily access their target (Wagner *et al*., 2003). Additionally, PNA probes are chemically more stable and resistant to enzymatic hydrolyses, such as nucleases and proteases (Demidov *et al*., 1994). Even more, PNA probes are ideal for targeting highly structured nucleic acids, such as rRNAs, since hybridization can be performed efficiently under low salt conditions. These conditions destabilize the secondary structures of the rRNA, resulting in improved access of the probe to the target sequences (Stefano & Hyldig-Nielsen, 1997).

1.3.2 Polymerase chain reaction (PCR)

Few techniques have transformed biological sciences as polymerase chain reaction (PCR). In 1974, Panet and Khorana described the principle of *in vitro* amplification of DNA. But PCR, as it is known, was developed by Mullis in the 1980s, who described it as a technique that "lets you pick the piece of DNA you are interested in and have as much of it as you want" (Garibyan & Avashia, 2013; Panet & Khorana, 1974). Without this technique, accomplishments as the Human Genome Project would not have been possible (Collins *et al*., 2003). PCR has a broad range of applications, not only in research for cloning, mutagenesis, and sequencing but also in various areas including forensics, to identify criminals, and medical diagnostics (Hentemann *et al*., 1990; Kasai *et al*., 1990; Nishiya & Imanaka, 1994). PCR has revolutionized medicine and is now used as a diagnostic tool either for familial identification either for the identification of microbial infections and genetic diseases like for instance the diagnosis of cancer (Hensel *et al*., 1991; Hentemann *et al*., 1990; Mackay, 2004; Tang *et al*., 1997). One of the most important applications of the classic PCR method is detection of pathogens. Considering that primers can be designed to identify specific DNA sequences conserved at various levels, detection can target the presence of a microbial genus, species, or strains (FDA-BAM, 2001). Indeed, PCR has been used to rapidly detect a variety of organisms and many studies have shown the efficiency of PCR for rapid detection of several species of bacteria (Allmann *et al*., 1995; Kaiser *et al.*, 2001).

1.3.2.1 Protocol

When PCR is used to detect the presence or absence of a specific DNA product, it is named qualitative PCR. PCR technique requires four primary components: the thermostable DNA polymerase, nucleotides, template DNA, and specific primers. The DNA polymerase links individual nucleotides together to form the PCR product. The nucleotides comprise the four nitrogenous bases found in DNA: adenine, thymine, cytosine, and guanine (A, T, C, G, respectively). The primers correspond to short DNA sequence-specific fragments complementary to the target DNA to be amplified (Garibyan & Avashia, 2013; Lynch & Brown, 1990). PCR is composed of repeating cycles of three consecutive steps with distinct temperatures, performed in a thermocycler. With each cycle, the number of copied DNA molecules doubles. Such amplification ability allows the detection of DNA from a single cell. The first PCR step (denaturation) consists of heating the sample mix above the melting point of the two complementary DNA strands of the target DNA, causing the separation of the double-stranded DNA (dsDNA). In the second step, temperature is lowered to allow the annealing of primers to the target DNA segments, if they are complementary sequences. In the third step, called elongation, temperature is raised to DNA polymerase optimum value to enable to extend the primers by adding nucleotides to the developing DNA strand. Finally, for analyzing the PCR product, the most frequently used method is agarose gel electrophoresis, which separates DNA products by their size. Regarding visualization of the PCR products, there are two main methods. Prior to PCR amplification, PCR primers or nucleotides can be labeled with fluorescent dyes (fluorophores); or the amplified DNA product can be stained with a chemical dye such as ethidium bromide, which intercalates between the two strands (Garibyan & Avashia, 2013; Lynch & Brown, 1990).

1.3.2.2 Advantages and withdraws

As previously mentioned, the molecular approach is a reliable procedure. This technique has proven to be highly rapid, sensitive and specific for routine microbial screening and monitoring of clinical, environmental and food samples. Being extremely sensitive, it only requires trace amounts of DNA to generate enough copies of a specific product for analysis, sequencing, and cloning (Garibyan & Avashia, 2013; Huq *et al*., 2012). Even more, direct PCR for microbial screening and monitoring of clinical, environmental and food samples allows the detection of VBNC bacteria which is a great improvement to microbiologists since many methods used today rely on the cultivation of the organism in question (Ramamurthy *et al.*, 2014). However, PCR also has limitations starting with the fact that it can detect DNA from dead cells which can lead to false positive results (Bonnin-Jusserand *et al.*, 2019). Due to being an extremely sensitive method, any contamination of the sample, even by trace amounts of DNA, can result in misleading results. In addition, primers can anneal nonspecifically to sequences that are similar, but not completely identical to the target DNA. Moreover, although at a very low rate, inappropriate nucleotides can be incorporated by the DNA polymerase (Garibyan & Avashia, 2013). Even more, several studies showed different causes of inhibition during direct PCR with food or environmental samples (Blackstone *et al*., 2003; Kaufman *et al*., 2004). Some components of food and culture media, such as fats and proteins, have been reported to interfere or inhibit the PCR (Rossen *et al*., 1992). One possible solution to reduce inhibitions in PCR reactions may be the use of diverse systems, during extraction, to purify the DNA. However, these greatly increase time and cost of the analysis, which results in a serious constraint to include their use in routine analyses (Perry *et al*., 2007).

1.4 Standard methods to detect *Vibrio* spp.

Despite advances in molecular methods for *Vibrio* spp. detection, health organizations recommend the use of standardized microbiological procedures such as the United States Food & Drug Administration's (FDA) Bacteriological Analytical Manual (FDA-BAM, 2004) and ISO 21872-1:2017 [International Organization for Standardization (ISO), 2017]. Even more, before implementing any method, national and international legislation must be checked in order to comply with specific regulations.

1.4.1 Current legislation for *Vibrio* spp. in seafood

The European Regulation No. 2073/2005 defines the microbiological criteria for food products produced and traded in Europe, but currently there are no specific regulatory microbiological criteria for *Vibrio* spp. in seafood products (Hartnell *et al.*, 2019). In 2010, Codex Alimentarius Commission (CAC) published the guidelines (CAC/GL 73–2010) on the application of general principles of food hygiene to the control of pathogenic *Vibrio* species in seafood. However, it did not provide any microbiological criteria. Consequently, governmental and non-governmental agencies produced guidelines for *Vibrio* spp. in post-processing or ready to eat seafoods. Some of these guidelines are presented in appendix in **Table 6.1** and **Table 6.2.** In their guidelines for microbiological contaminants in seafoods, the FDA, in the United States of America, has a zero-tolerance for *V. vulnificus* and for *V. cholerae* O139 and O1, in ready to eat products. For *V. parahaemolyticus*, the FDA has a limit of acceptability of 10^4 CFU per g. Distinctly, the United Kingdom considers that between 20 and 10^3 CFU per g it represents a moderate risk for human health and, therefore, the satisfactory level is established at bellow 20 CFU per g. In general, where numerical standards or guidelines have not been defined for a pathogen, food microbiology laboratories usually test for presence/absence of a pathogen in 25 g. The "absence in 25 g" demonstrates compliance with zero-tolerance plans. In products that have an allowable tolerance, such as the specifications given for *V. parahaemolyticus,* a Most Probable Number (MPN) test may be

applied to obtain the required precision and confidence that levels do not exceed the established values [Food and Agriculture Organization of the United Nations (FAO) & World Health Organization (WHO), 2017]. ISO/NP TS 21872-2, still in proposal, is dedicated to the quantitative determination in seafood of total and potentially enteropathogenic *V. parahaemolyticus* using nucleic acid hybridization.

1.4.2 FDA-BAM standard method

Concerning United States Food & Drug Administration's (FDA), the Chapter 9 of the FDA-BAM (2004), describes isolation methods for *V. cholerae,* including sample preparation and the enrichment step in Alkaline Peptone Water (APW), followed by the isolation step. The last step, biochemical tests, allows to assure that it is or not *V. cholerae* and the production of Cholera Toxin (CT). Equivalent methods for the detection of *V. parahaemolyticus* and *V. vulnificus* are also described. For *V. parahaemolyticus* and *V. vulnificus* enumeration, the MPN procedure is the most frequently used in laboratories. Some molecular methods have been introduced, since FDA-BAM (2004) recommends using a PCR as a final step for the identification step, also contributing to characterization of the pathogenicity of the isolates.

1.4.3 ISO method

Regarding the International Organization for Standardization method, ISO/TS 21872-1:2007 - "Microbiology of food and animal feeding stuffs - Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. - Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholera"* and ISO/TS 21872-2:2007 - "Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholera"* were combined in just one, ISO 21872-1:2017, dedicated to *V. cholerae, V. parahaemolyticus* and *V. vulnificus.* ISO 21872-1:2017 comprises four main microbiological steps: enrichment, isolation, identification and confirmation (ISO, 2007; ISO, 2017). ISO 21872-1:2017 requires two successive enrichment steps done in Alkaline Saline Peptone Water (ASPW) with similar incubation times but with different temperatures depending upon the target bacterial species or sample matrix state. The isolation and identification steps consist of the plating of the enrichment broths on selective Thiosulfate Citrate Bile and Sucrose (TCBS) agar and a second selective medium chosen by the laboratory.

TCBS agar is a highly selective medium for *Vibrio* spp. due to bile salts, thiosulfate, citrate, and a high pH of 8.6. A derivative of bile salts, Oxgall, and sodium citrate inhibit the growth of most Gram-positive bacteria, such as enterococci, and high concentrations of thiosulfate and citrate in combination with the strong alkalinity inhibits most Enterobacteriaceae growth. Sodium thiosulfate also represents a source of sulfur, which in combination with ferric citrate detects the production of hydrogen sulphide (H_2S), resulting in black colonies. Since *Vibrio* spp. do not produce H_2S, these black colonies can be excluded as possible *Vibrio* spp. (Corry *et al.*, 2011; Muyzer & Stams, 2008; Nicholls *et al*., 1976; Sigma-Aldrich, n.d.). Additionally, species that ferment sucrose are detected by the mixed pH indicators thymol blue and bromothymol blue that change their color to yellow, when acid is formed. However, even if TCBS agar is selective, a few sucrose positive *Proteus* strains may grow to form yellow colonies like *Vibrio*. Furthermore, coliform bacteria that cannot metabolize sucrose may also grow (Merckmilipore, n.d.; Nicholls *et al*., 1976; Sigma-Aldrich, n.d.). Even more, isolates of *Pseudomonas* and *Aeromonas* species may form, occasionally, blue or green colonies (Sigma-Aldrich, n.d.). As shown in **Figure 1.4** there are two typical morphologies for colonies of *Vibrio* spp. on TCBS agar. Colonies with 2 - 3 mm in diameter, smooth, yellow (sucrose positive) are characteristic of *V. cholerae* while smooth, green (sucrose negative) are typical of *V. parahaemolyticus* or *V. vulnificus* (ISO, 2017).

V. cholerae *V. parahaemolylicus* *V. vulnificus*

Figure 1.4 - Typical morphologies for colonies of *Vibrio* spp. on TCBS agar. Adapted from Nakashima *et al*. (2007).

Limitations of the TCBS comprise results that are often difficult to interpret and cannot precisely distinguish between *Vibrio* spp. (Hara-Kudo *et al.*, 2001; Yamazaki *et al.*, 2011). Consequently, the use of a second selective medium, chosen by the laboratory, is required. Some examples for *V. cholerae* and *V. parahaemolyticus* are Soya Peptone Triphenyl Tetrazolium Chloride agar (TSAT) and Sodium Dodecyl Sulfate Polymyxin Sucrose (SDSPS) agar. *V. vulnificus* is also isolated using modified Cellobiose-Polymyxin B-Colistin (mCPC) agar (Bhunia, 2018; ISO 2007).

More recently, ISO 21872-1:2017 suggests the use of a chromogenic agar as the second selective media. In fact, advances in chromogenic media allow the detection of species-specific enzymes, offering rapid identification directly on the plates. In general, chromogenic media demonstrate superior sensitivity and specificity compared to conventional selective media (Eddabra *et al*., 2011). Even more, some chromogenic agars are considered to be less inhibitory to *Vibrio* spp. than TCBS and to facilitate identification of the presumptive pathogenic species, offering better discrimination between species and from other bacteria (FAO & WHO, 2017). A variety of chromogenic media were developed to detect *Vibrio* spp. such as Vibrio ChromoSelect® Agar (Sigma-Aldrich) that merely differentiates *V. cholerae* and *V. parahaemolyticus*, not mentioning *V. vulnificus* (Sigma-Aldrich, n.d). Contrarily, CHROMagar™ Vibrio (CHROMagar™) and ChromID® Vibrio (BioMérieux) were developed for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* detection (BioMérieux, n.d.; CHROMagar™, n.d.) However it has not been an extensive evaluation of the performance of these media, some assays were performed.

Concerning ChromID® Vibrio, pink colonies correspond to *V. parahaemolyticus* and blue to green colonies are characteristic of *V. cholerae* and *V. vulnificus* (BioMérieux, n.d.). In a study with human clinical specimens, ChromID® Vibrio displayed a sensitivity to *V. cholerae* and *V. parahaemolyticus* equivalent to TCBS medium but was two times more specific, which translates into a lower number of false positives compared to TCBS (Eddabra *et al.* 2011). Another assay showed that, although TCBS was much less efficient than ChromID® Vibrio, it allowed isolation of *V. parahaemolyticus* in five samples (12.5 % of the positive ones), while ChromID® Vibrio failed to detect. Consequently, the use of only one specific chromogenic medium, replacing the two selective media prescribed in the protocol was not recommend (Rosec *et al*., 2012).

The medium CHROMagar™ Vibrio uses high pH (9.0) as a selective factor for *Vibrio* spp. and discriminates among strains based on differences in their ability to metabolize chromogenic substrates. Some of the medium components are known but the chromogenic mixture, responsible for the color change is confidential. As shown in **Figure 1.5**, on CHROMagar™ there are two typical colonies morphologies for the three species targeted: typical colonies of *V. parahaemolyticus* are mauve, and typical colonies of *V. cholerae* and *V. vulnificus* appear green blue to turquoise blue. *V. alginolyticus* remains colorless, avoiding any interference with the targeted species (CHROMagar™, n.d.).

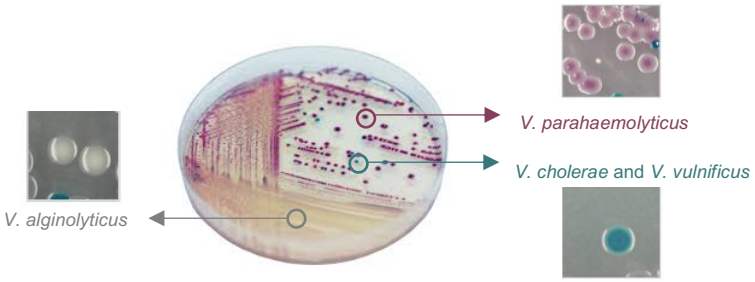


Figure 1.5 - Typical morphologies for colonies of *V. parahaemolyticus* and *V. vulnificus*/*V. cholerae* on CHROMagar™ Vibrio. Adapted from CHROMagar™ (n.d).

Two studies investigated the TCBS and CHROMagar™ accuracy and specificity for isolating *V. parahaemolyticus* from seafood samples. Both studies indicated that CHROMagar™ is more accurate and specific than TCBS (Di Pinto *et al*., 2011; Hara-Kudo *et al*., 2001). Contrarily, Nigro and Steward (2015) revealed that, for other environmental samples analyzed, there was no significant difference in the false positive rates for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* for the two media. However, neither of these previous tests was done with microalgae cultures. Hence, the range of false positives obtained can not be directly compared to the range expected with microalgae cultures which could influence the efficiency of both media.

Even on chromogenic media, presumptive *Vibrio* spp. colonies require additional confirmation steps to confirm their identity and rule out false positives. Firstly, a subculturing step onto non-selective media is required since it is critical that pure colonies are isolated before proceeding to any phenotypic characterization steps. Secondly, growth on TCBS is not suitable for the oxidase test, interfering with results. This confirmation involves a series of tests to further characterize the isolate, starting with a test for oxidase, Gram staining as well as microscopic observation for motility. The oxidase positive and Gram-negative colonies which give a positive result in the motility test, if examined, should be retained for further confirmation. In ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 further confirmation consisted of biochemical tests comprising detection of ornithine decarboxylase, L-lysine decarboxylase, arginine dihydrolase, β-galactosidase and indole, a test for halotolerance as well as a test with Saline Triple sugar Iron (TSI) to determine glucose fermentation, hydrogen sulfide formation and lactose and/or sucrose utilization. More recently, in ISO 21872-1:2017 the biochemical tests were revised and detection of ornithine decarboxylase and the test with TSI were eliminated. The main novelty of ISO 21872-1:2017 is that it presents PCR, in addition or as an alternative to biochemical tests. The PCR is a useful alternative to labor-intensive biochemical tests, which are occasionally difficult to interpret. Primers for non-pathogenic *V. parahaemolyticus* identification target *toxR* and for pathogenic strains genes encoding for TDH and TRH are targeted. For *V. cholerae* the target region for PCR is the 16S - 23S rRNA intergenic spacer region *prVC* and for *V. vulnificus* the haemolysin region (*vvha*) (ISO, 2017).

In addition to the official ISO 21872-1:2017 method, where PCR can be used as a way to confirm the identity of the colonies, detection using PCR can be applied directly to samples as well as to samples following pre-enrichment steps (Huq *et al.*, 2012). The latest is mentioned in the ISO 21872-1:2017 as a screening method. In fact, this method has been shown to be more successful in detecting *V. parahaemolyticus* in at least double the number of samples than the plate culture method (Blanco-Abad *et al.*, 2009; Rosec *et al*., 2012). Even more, direct PCR to samples allows the detection of VBNC which, as previously mentioned, present an improvement to microbiologists analysis because many methods used today rely on the cultivation of the organism (Ramamurthy *et al.*, 2014). However, PCR can detect DNA from dead cells which can lead to false positive results. Hence, no method is ideal and the restrictions of both methods must be taken into account when evaluating results (Bonnin-Jusserand *et al.*, 2019).

1.5 Possible treatment to eliminate *Vibrio* spp.

As stated on the bulletin of the World Health Organization (Nair & Narain, 2010), De in (1959) discovered that cholera is caused by a potent exotoxin (cholera enterotoxin), currently known as Cholera toxin (CT), affecting intestinal permeability and demonstrated that bacteria-free culture filtrates were enterotoxic. The fact that, even in the absence of the bacteria, the filtrate is still able to cause disease is not in accordance with the information provided by Hernández-Cortez *et al.* (2017) where *V. cholerae* is described as a microorganism that can cause toxico-infections by the production of toxins after the consumption of the microorganisms and not by the intoxication caused by the consumption of merely the bacterial toxin. Further studies need to be performed to corroborate that filtrates of *V. cholerae* can be enterotoxic. Considering the serious hazard that *V. cholerae, V. parahaemolyticus* and *V. vulnificus* present to public health, allied with the fact that the treatments commonly used to eliminate *Vibrio* spp. from seafood products, such as heat treatment or irradiation, cannot be applied to microalgae biomass, this excludes the possibility of implementing a treatment to eliminate *Vibrio* spp. if detected. Therefore, if *Vibrio* spp. were to be detected in the microalgae biomass, these products should not be commercialized and the National Health Organization should be notified of the *Vibrio* spp. detected (European Comission, 2001).

1.6 Thesis objectives

Considering the increasing microalgae production, the main goal of this work is to implement internal methods to detect *Vibrio* spp. in microalgae biomass according to the ISO 21872-1:2017, satisfying the customers' request for a standard method. The final goal is to infer if it offsets to implement the method at A4F's laboratory or if it is preferable to ask a reference laboratory to perform the analyses. To answer this question, the work comprises the following specific objectives:

- To implement biosafety measures required to work with risk II microorganisms at A4F's laboratory;
- To infer the efficiency of the selective enrichment broth (ASPW) and the main selective differential agar medium (TCBS), that consequently affect the number of steps required to conclude the absence of *Vibrio* spp., and to rule out false positives;
- To determine the efficiency of CHROMagar™ as the second selective differential medium;
- To test PCR method as the confirmation step, as an alternative to biochemical tests;
- To compare direct PCR to the enrichment broths, additionally to the ISO 21872-1:2017 method, to the results obtained according to the official method;
- To check if the method can detect *Vibrio* spp. or if a high number of false positives can mask the true positives leading to false conclusions. To assess this, samples artificially contaminated with known concentrations of *Vibrio* spp., usually known as spiked samples, were analyzed according to the method established;
- To understand if it offsets to perform the method at A4F rather than asking a reference laboratory to do the analyses. To conclude this, a financial analysis of the method is performed.

Additionally, considering the resources available, the second goal is to use FISH probes provided by Biomode SA as a method to detect *Vibrio* spp. directly in microalgae cultures.

Chapter 2 - Materials and Methods

The equipment and reagents/solutions used in the experiments described along this section are presented in appendix **Table 6.3** and **Table 6.4.**

2.1 Biosafety guidelines

Vibrio is a risk II microorganism, a pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited. Risk II microorganisms represent a moderate individual risk and low community risk (Council of the European Union, 2000). Therefore, previously to handling *Vibrio* spp. for any experiments, a Biological Agent Reference Sheet for the agents *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* was done to assure all the required biosafety measures would be fulfilled. Biosafety encompasses all aspects of containment to prevent any exposure and accidental release of infectious biological material (Thomson, 2012). The planning and implementation of safety practices must be part of every laboratory's routine activities in order to minimize any risks for human health, preventing laboratory-acquired infections, and the environment, eliminating the spread of contamination. Risk II microorganism manipulation involve laboratories requiring containment level 2 (CL2). Hence, A4F's laboratory had the requirements to work with *Vibrio* spp. according to the guidelines presented by Best *et al.* (2004). *Vibrio* spp. primary hazards are associated with ingestion, accidental parenteral inoculation (*e.g.* needle stick) and mucous membrane route. Generally, risk group II agents are not transmitted by airborne routes, but care must be taken to avoid aerosols or splashes (Best *et al.*, 2004; U.S. Department of Health and Human Services, 2009).

Beyond the commonly adopted laboratory practices, additional care had to be taken when working with risk II microorganisms. All procedures were done inside a class II biological safety cabinet with a downward laminar airflow that provides protection to the worker, the environment and the experiment (WHO, 2004). Two pairs of Nitrile gloves were used for all procedures and before removal from biosafety cabinet, containers were sealed with parafilm and material wiped with 1:10 bleach dilution and/or 70 % ethanol. These concentrations were chosen after meticulous research in this topic, presented below. Disinfectants exploit two standard methods of action: either by destroying the lipid membrane of a microorganism which results in the leaking of cell material (*e.g.* ethanol) or by destroying proteins and enzymes essential for the survival of the microorganism (*e.g.* chlorine) (Flanders Interuniversity Institute for Biotechnology, 2004). Chlorine is commonly sold as bleach, an aqueous solution of sodium hypochlorite (NaOCl). The concentration of a general all-purpose laboratory disinfectant should be 1 g L^{-1} of available chlorine. A stronger solution, 5 g L^{-1} available chlorine, is recommended for dealing with biohazardous spillage. Domestic bleach contains 50 g L^{-1} available chlorine and should be diluted 1:50 or 1:10 to obtain final concentrations of 1 g L^{-1} and 5 g L^{-1} , respectively (WHO, 2004). Ethanol (ethyl alcohol, C_2H_5OH) should be used at 70 % (V V^{-1}) in demineralized water. Higher or lower concentrations may not be as germicidal (WHO, 2004). Therefore, 1:10 household bleach and 70 % ethanol solutions were chosen to decontaminate materials and work surfaces.

After finishing work in the safety cabinet, according to A4F's internal protocol, UV decontamination for 30 min was applied with all the remaining material inside. Then, the biohazard bags to dispose were placed in the biological hazard waste bin and reusable material was duly identified and placed to decontaminate. The biosafety cabinet surfaces were cleaned with 1:10 bleach dilution and then with

70 % ethanol and further UV light decontamination for 15 min was applied. All the equipment used was identified with the sign “biological hazard” and all infectious materials and all contaminated equipment or apparatus was decontaminated before being washed and stored or discarded.

Regarding biological waste management, the information mentioned by Flanders Interuniversity Institute for Biotechnology (2004) was taken into account. A distinction was made between solid, pasty and fluid waste as presented in **Table 2.1.**

Table 2.1 - Different types of waste (Flanders Interuniversity Institute for Biotechnology, 2004).

Type of waste	Inactivated	Chemically polluted	Destination
Solid/pasty waste	+	-	=> residual waste
Solid/pasty waste	+	+	=> chemically polluted waste
Solid/pasty waste	-	+ or -	=> hazardous medical waste
Fluid waste	+	-	=> waste water
Fluid waste	+	+	=> chemically polluted waste
Fluid waste	-	+ or -	=> hazardous medical waste

For inactivation, steam autoclaving was the preferred method for all decontamination processes, being the most reliable procedure for ensuring the destruction of microorganisms. According to Flanders Interuniversity Institute for Biotechnology (2004), steam autoclaving at 121 °C for 20 min is the appropriate inactivation method for some relevant microorganisms such as *E. coli*, *Lactobacillus*, *Salmonela* and *Aspergillus*. Hence the chosen combination to apply in A4F’s was 120 °C for a minimum of 20 min. Although, since the time is also based on the size of the load and the organism being autoclaved, to ensure the effectiveness of the process, a 40 min cycle was performed. As stated in **Table 2.1**, considering that the waste was not chemically polluted, after inactivation, it was disposed of as residual waste and the reusable material was safely washed and stored. Regarding treatment and disposal of liquid waste, it occurred by autoclave and disposal to the sanitary sewer via the laboratory sink according to Flanders Interuniversity Institute for Biotechnology (2004). In conclusion, safety practices mentioned above were strictly carried out in order to minimize any risks for human health and the environment.

2.2 *Vibrio* spp. positive controls maintenance

Considering A4F's laboratory specialization in the microalgae field, there were no previous protocols for maintenance of bacterial cultures. Firstly it was necessary to establish methods for short-term and long-term maintenance of the *Vibrio* spp. positive controls.

The protocols implemented for cryopreservation and maintenance of *Vibrio* spp. were adapted from Martinez *et al.*, (2010) that recommended Luria Broth (LB) [10 g tryptone, 5 g yeast Extract, 5 g sodium chloride (NaCl) to 1 L of deionized, distilled water] at 37 °C as standard laboratory conditions for *Vibrio cholerae* growth. The methods with small modifications were tested and successfully applied for the three species (*Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus).* Martinez *et al.*, (2010) suggested using neutral pH 7.0 for maintenance and growth of *V. cholerae*, although after some tests performed, pH 8.0 revealed to be the optimum pH for the growth of the three species together, as previously mentioned by Bhunia (2018). Finally, considering broth cultures grow best with aeration, a shaker was required for optimal growth (Martinez *et al.*, 2010).

2.2.1 Growth from a frozen stock

Growth from a frozen stock is an essential starting point for any experiment. *Vibrio* spp. should be preserved for long-term in frozen stocks in 30 % glycerol stored at -80 °C and strains that are used frequently should be re-frozen after a single passage to have a stock (Martinez *et al.*, 2010). Due to the unavailability of a -80 °C freezer, a -70 °C freezer was used. To grow from a *Vibrio* sp. frozen stock, an LB plate (15 g Agar to 1 L of LB) was inoculated using a sterile inoculating loop (10 µL). The plates were incubated at 37 °C for 16 - 24 h to obtain isolated colonies. It is also important to transfer a freezer stock onto an agar plate and not directly into liquid medium (Martinez *et al.*, 2010).

2.2.2 Growth in liquid medium and preparation of frozen stocks

Using a sterile inoculation loop, a single colony was inoculated into 5 mL liquid LB in a 15 mL tube, vortexed for 2 s and incubated at 37 °C with shaking (200 rpm) for 15 - 16 h (overnight). A volume of 500 µL from the overnight bacterial culture was added to 750 µL of 50 % glycerol solution and placed into a freezer vial. The suspension was well mixed by vortexing and immediately placed at -70 °C.

2.2.3 Maintenance of *Vibrio* spp. positive controls

V. parahaemolyticus and *V. cholerae* grown in LB plates were viable when stored at 4 ºC. Although, *V. vulnificus* lost viability when stored at 4 °C after just 24 h. This occurrence was in accordance with Johnston and Brown (2002) that verified that at 4 °C cells became non-culturable over time. Therefore, *Vibrio* spp. grown in LB plates were stored at 15 °C, for one week, in the appropriate biohazard shelf.

2.3 FISH

In addition to the official ISO 21872-1:2017 method, detection using FISH was tested as a method to detect *Vibrio* spp. directly in microalgae cultures. The detection of *Vibrio* spp. in the cultures would lead to the disposal of these contaminated cultures in order to avoid further costs in cultures that could not be commercialized.

2.3.1 Correlation between Optical Density (OD) and CFU mL^{-1}

To infer the detection limit of the FISH method it was necessary to previously obtain a correlation between $OD_{600\ nm}$ and CFU mL^{-1}. To do so, a single colony of *V. parahaemolyticus* was inoculated into 5 mL LB pH 8 (Martinez *et al.*, 2010). The culture was incubated in an orbital incubator overnight at 37 °C with vigorous shaking, 200 rpm. The following day, a sample was taken and diluted to a final $OD_{600\ nm}$ of 0.05 to 0.06 in a total volume of 40 mL LB, and incubated at 37 °C with shaking (200 rpm). The OD was measured every 30 min until reaching the stationary phase. During the exponential phase, 3 samples (0.5 h; 1 h and 1.5 h of incubation) were collected, and several serial dilutions of 1:10 were prepared. Afterward, 100 µL of each was inoculated in LB plates (triplicates) and after 16 h of incubation at 37 °C, CFU were counted in the plates presenting between 30 and 300 colonies (Maier *et al.*, 2000).

2.3.2 FISH - Hybridization procedure on suspension

The three probes used for the detection of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* were developed by Biomode SA, allowing the detection of these species under fluorescence microscopy or cytometry. These probes were connected to a fluorochrome from the cyanine family, Cyanine-3 (Cy3), that has an excitation maximum at 554 nm and an emission maximum at 568 nm (**Figure 2.1**). Biomode SA tests concluded that the probes used in this procedure present theoretical values of 100 % sensitivity and specificity at 61 °C and a limit of detection of 10^5 - 10^6 CFU mL^{-1}.

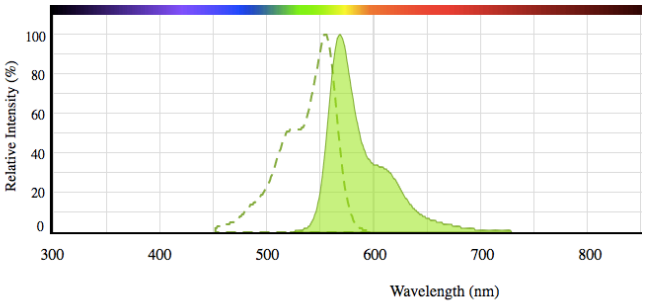


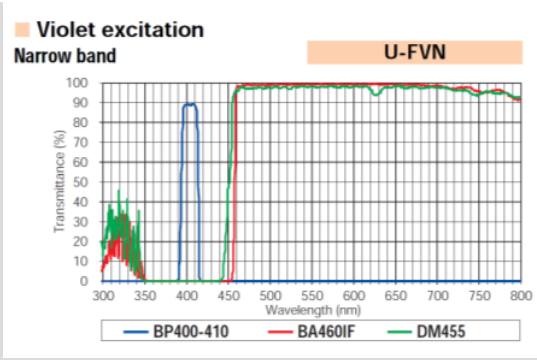
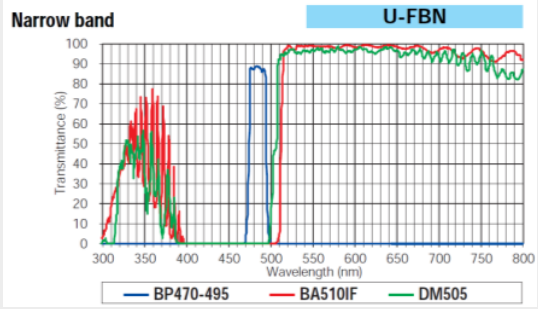
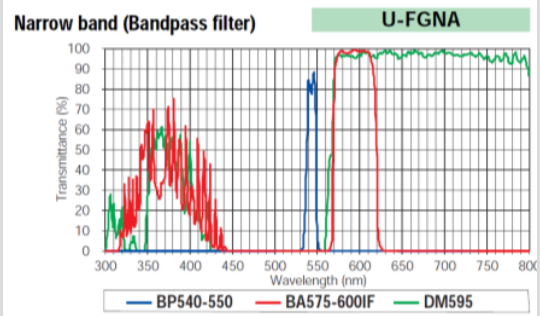
Figure 2.1 - Fluorescence spectral profile for fluorochrome Cy3. ---- : excitation; —— : emission. Adapted from AAT Bioquest ® (n.d.).

The procedure applied was adapted from the procedure developed for the *Vibrio* spp. probes by Biomode SA with slight modifications. Firstly, 50 µL of liquid culture was pelleted by centrifugation at 14129 x *g* for 15 min, resuspended in 20 µL of 4 % (V V^{-1}) formaldehyde, and fixed for 1 h at room temperature. The fixed cells were centrifuged at 14129 x *g* for 15 min and resuspended in 25 µL of 50 % (V V^{-1}) ethanol, and incubated for 30 min at -20 °C. After centrifuged at 14129 x *g* for 15 min, the cells were incubated in a thermal cycler at 61 °C for 1 h with 50 µL Biomode PNA probe (200 nM in hybridization solution). After hybridization, cells were centrifuged at 14129 x *g* for 15 min, resuspended in 200 µL of washing solution [5 mM tris Base, 15 mM NaCl and 1% (V V^{-1}) triton-X] and incubated at 61 °C for 30 min. Washed suspension was pelleted by centrifugation and resuspended in 100 µL of

distilled autoclaved water. Finally, 20 µL of the cell suspension was spread on a microscope slide for observation under fluorescence microscopy on the Olympus BX53 microscope, equipped with the most suitable filter cube for the fluorophore Cy3, available at A4F. This filter cube U-FGNA includes an excitation filter (excitation: 540-550 nm), a dichromatic mirror and an emission filter (emission: 575-600 nm). **Table 2.2** compiles the information given in the technical specifications data sheet (UIS2 fluorescence mirror units) by Olympus (n.d.). As seen in **Table 2.2** and **Figure 2.1**, the cube available, U-FGNA, is not the best option available in the market for Cy3, once the emission filter does not comprise the maximum wavelength emission for Cy3, 568 nm. Even though, at 575 nm the probe still presents 80 % to 90 % of signal intensity which enables the use of this filter cube for the fluorophore Cy3.

Table 2.2 - Filters available for A4F's Olympus microscope and fluorescence filter spectral profile for each filter.

— BP: Excitation filter; — BA: Emission filter; — DM: Dichromatic mirror (Olympus, n.d.).

Position	Name	Fluorescence filter spectral profile
2	U-FVN	
3	U-FBN	
4	U-FGNA	

For the FISH method, positive and negative controls were considered. Positive controls consisted of the target *Vibrio* sp. with the specific probe while negative controls, for each reaction, contained the sample with hybridization solution, minus the probe.

During protocol optimization, one of the problems faced was the autofluorescence of chlorophyll in the microalgae (Schulze *et al.,* 2011). To resolve this problem, Sudan Black B was tested as an

autofluorescence reduction treatment. This way, an additional step was applied subsequently to incubation for 30 min at -20 °C. The fixed-cell aliquot was pelleted by centrifugation at 14129 x *g* for 15 min and resuspended in 30 µL of 0.1 % (m V^{-1}) Sudan Black B in 70 % (V V^{-1}) of ethanol, and incubated for 20 min at room temperature in the dark. Then, the samples were washed 3 times by centrifugation and resuspension in phosphate-buffer saline (PBS) containing 60 mM NaCl, 1 mM KCl, 0.5 mM KH_2PO4 and 3 mM $Na_2HPO_42H_2O$.

2.4 Determination of the Detection Limit

According to Shrivastava and Gupta (2011), after a comparison of various regulatory authorities, there is no universal definition of Limit Of Detection (LOD) and method for its determination. The International Conference on Harmonization (ICH) refers to the limit of detection as the "Lowest amount of analyte in the sample, which can be detected but not necessarily quantitated under stated experimental conditions" (Shrivastava & Gupta, 2011).

To determine the limit of detection of the FISH method, using Sudan Black B to inhibit the autofluorescence of *Nannochloropsis* sp.*,* samples collected from a photobioreactor at A4F's facilities were artificially contaminated with known concentrations of *V. parahaemolyticus*. To do so, *V. parahaemolyticus* was grown for 4.5 h until late stationary phase to reach cells with low rRNA content, which is expected for environmental bacteria (Schauer *et al.,* 2012). Serial dilutions of 1:10 were prepared and afterward diluted 1:10 in *Nannochloropsis* sp. culture.

The hybridization procedure on suspension with Sudan Black B as an autofluorescence reduction treatment was then applied to the spiked samples to infer the limit of detection. Positive and negative controls were considered. Negative controls for each sample consisted on the hybridization solution, without the probe, while the positive control consisted of the original culture of *V. parahaemolyticus*. Additionally, *Nannochloropsis* sp. culture from photobioreactor was tested as a control to confirm the absence of *V. parahaemolyticus* contamination in the original culture.

2.5 ISO 21872-1:2017 Method

The current reference method for the detection of potentially enteropathogenic *Vibrio* spp. is the ISO 21872-1:2017. Though, it was not possible to promptly acquire the ISO 21872-1:2017 dedicated to the detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* (ISO, 2017). Hence, preliminary tests were performed according to the ISO/TS 21872-1:2007 – "Microbiology of food and animal feeding stuffs - horizontal method for the detection of potentially enteropathogenic *Vibrio* spp. - Part 1: detection of *V. parahaemolyticus* and *V. cholera"* and ISO/TS 21872-2:2007 – "Part 2: detection of species other than *V. parahaemolyticus* and *V. cholera",* lacking a few requirements such as the second selective medium and the confirmation step (ISO, 2007). Later, the complete ISO 21872-1:2017 method was tested. Considering both methods only present slight differences, in this section both methods are presented in parallel, pointing out the variations (**Figure 2.2, Figure 2.3** and **Figure 2.4**) which can be compared to the official diagram of procedure, in appendix, presented in ISO/TS 21872-1:2007 (**Figure 6.1**), ISO/TS 21872-2:2007 (**Figure 6.2**) and ISO 21872-1:2017 (**Figure 6.3**). Regarding the sampling, microalgae paste samples were collected from a photobioreactor, provided by Algae for Future (A4F).

2.5.1 Positive controls

The positive controls of *V. vulnificus*, *V. parahaemolyticus* and *V. cholera* were obtained from the Faculty of Engineering from University of Porto. The positive controls plates were prepared according to A4F's method. To confirm the efficiency of the sample's first enrichment, in parallel to the sample analysis, the same method was applied to the positive controls. Each *Vibrio* strain, picked from an LB plate, was used separately for the inoculation of 1 mL of medium used for the microalgae cultivation and the suspension was diluted 1:100 using the same medium. A new dilution of 1:10 was done in ASPW (pH 8.6, containing 20 g Peptone and 20 g NaCl to 1 L of deionized distilled water) and incubated at 37 °C for 6 ± 1 h. From both dilutions TCBS agar (89.1 g of mixture to 1 L of deionized distilled water. Mixture contained 5 g Yeast Extract, 10 g Peptone, 10 g Sodium Citrate, 10 g Sodium Thiosulfate, 8 g Oxgall, 20 g Sucrose, 10 g NaCl, 1 g Ferric Citrate, 0.04 g Thymol Blue, 0.04 g Bromothymol Blue, 15 g Agar) and CHROMagar™ plates (containing 8 g Peptone and Yeast extract, 51.4 g Salts, 0.3 g Chromogenic mix, 15 g Agar to 1 L of deionized distilled water) were inoculated with a 1 µL or 10 µL sampling loop and incubated at 37 °C for 24 ± 3 h.

Figure 2.2 presents the main differences between the preliminary tests according to the ISO/TS 21872-1:2007 or ISO/TS 21872-2:2007 and the ISO 21872-1:2017 method latter applied.

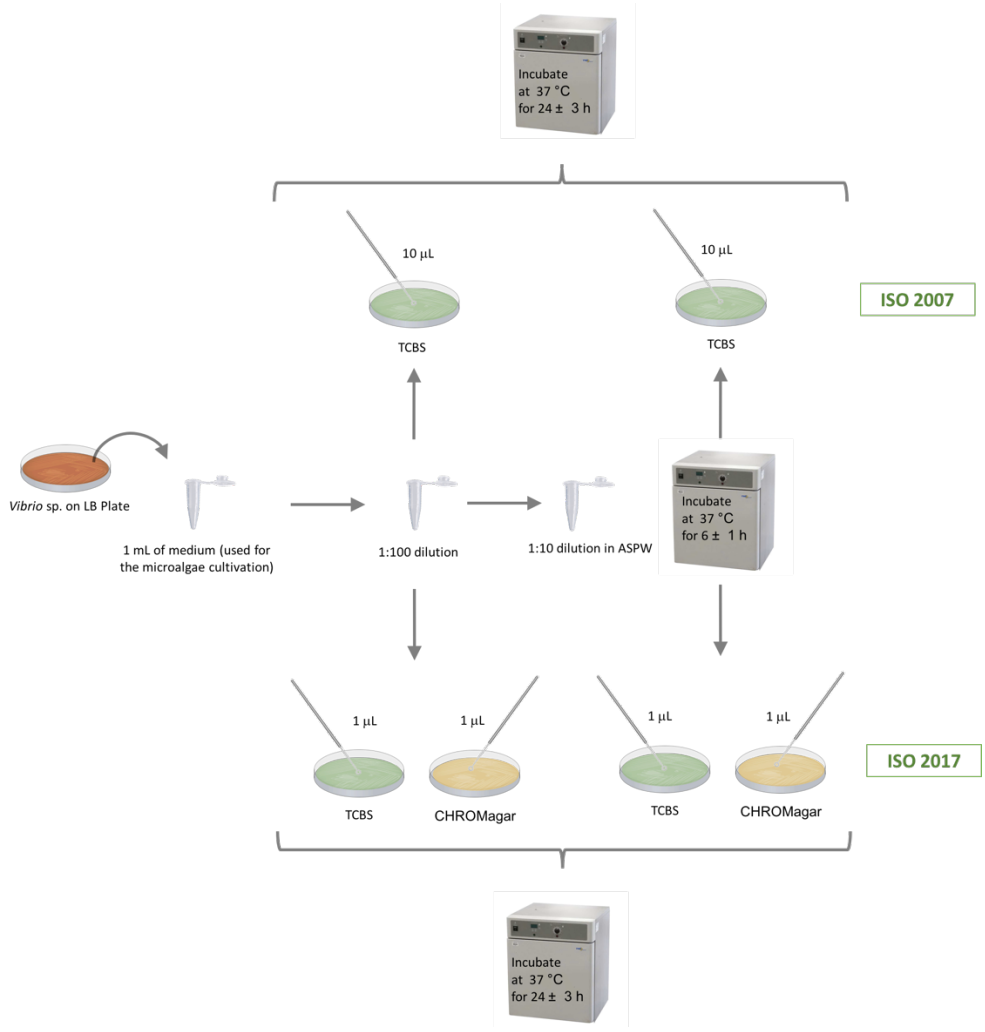


Figure 2.2 - Preparation of the positive controls (A4F's method).

2.5.2 Detection of *Vibrio* spp. in the sample

2.5.2.1 First selective enrichment

The biomass paste, previously tested as negative for *Vibrio* spp. by a reference laboratory, was diluted 1:9 (m V^{-1}) using ASPW, homogenized and incubated at 37 °C for 6 ± 1 h. The reference values consist on 25 g of sample to 225 mL of ASPW (ISO, 2017). Given that the paste is a frozen product, the incubation temperature for *V. parahaemolyticus* and *V. cholerae* was the same for the detection of *V. vulnificus*.

2.5.2.2 Second selective enrichment

From the culture obtained in the first enrichment, 1 mL was taken and added to a tube containing 10 mL of ASPW. For detection of *V. vulnificus*, the tube was incubated at 37 °C for 18 ± 1 h and for detection of *V. parahaemolyticus* and *V. cholerae* incubated at 41.5 °C for 18 ± 1 h. According to the ISO 21872-1:2017, samples should always be taken from the surface.

2.5.2.3 First isolation

With a 1 µL or 10 µL sampling loop, the surface of TCBS agar plates (duplicates) and CHROMagar™ plates (duplicates) were inoculated with the culture obtained after the first enrichment. The plates were incubated at 37 °C for 24 ± 3 h to allow the development of well-isolated colonies.

2.5.2.4 Second isolation

For the detection of *Vibrio vulnificus*:

With 1 µL or 10 µL sampling loop, the surface of TCBS agar plates (duplicates) and CHROMagar™ plates (duplicates) were inoculated with the culture obtained after the second enrichment incubated at 37 °C for 18 ± 1 h. The plates were incubated at 37 °C for 24 ± 3 h.

For the detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*:

With 1 µL or 10 µL sampling loop, the surface of TCBS agar plates (duplicates) and CHROMagar™ plates (duplicates) were inoculated with the culture obtained after the second enrichment incubated at 41.5 °C for 18 ± 1 h. The plates were incubated at 37 °C for 24 ± 3 h.

After each incubation period, the plates were examined for the presence of typical colonies of presumptive pathogenic *Vibrio* spp.

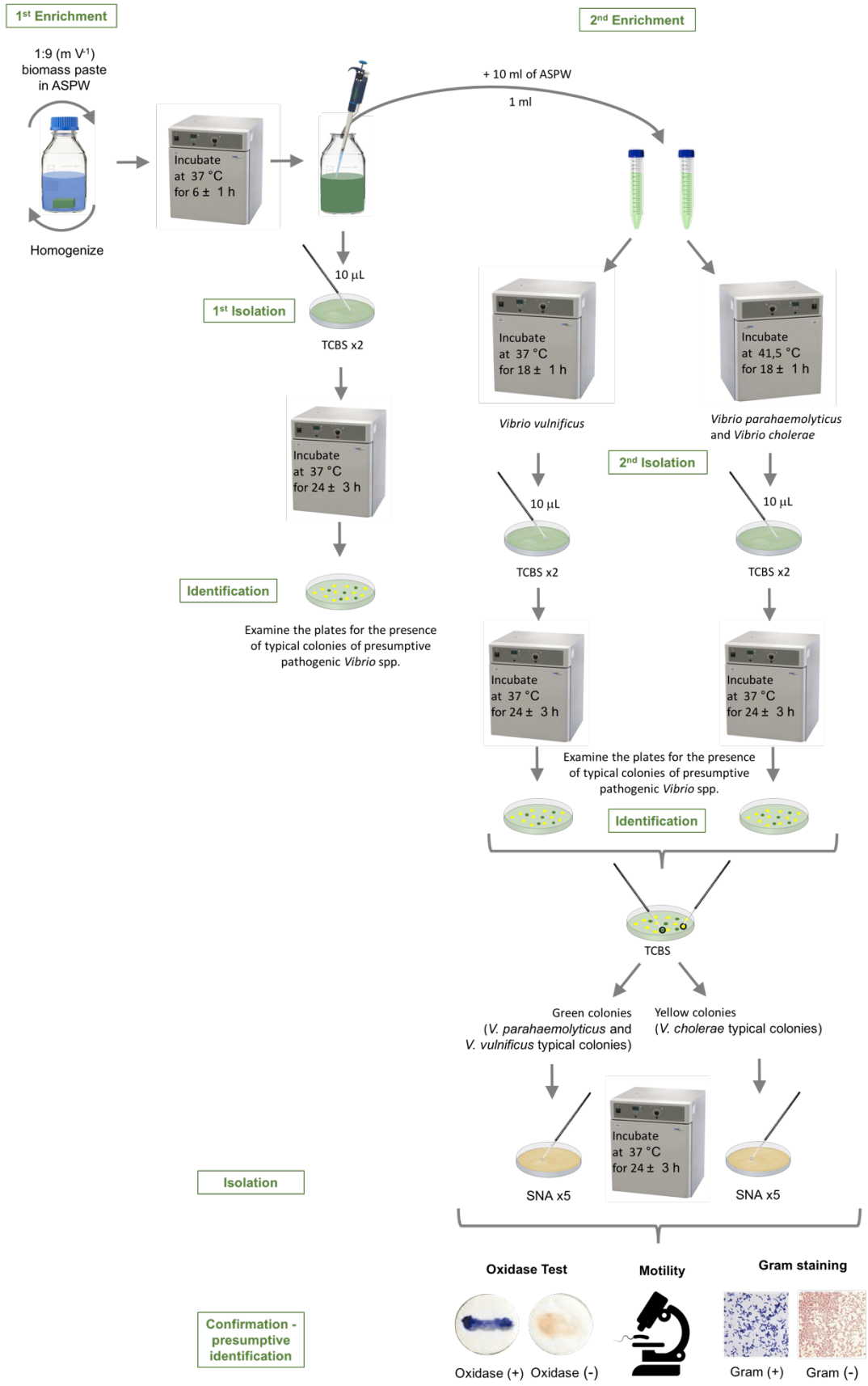


Figure 2.3 - Diagram of the method for the preliminary tests, according to the ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007.

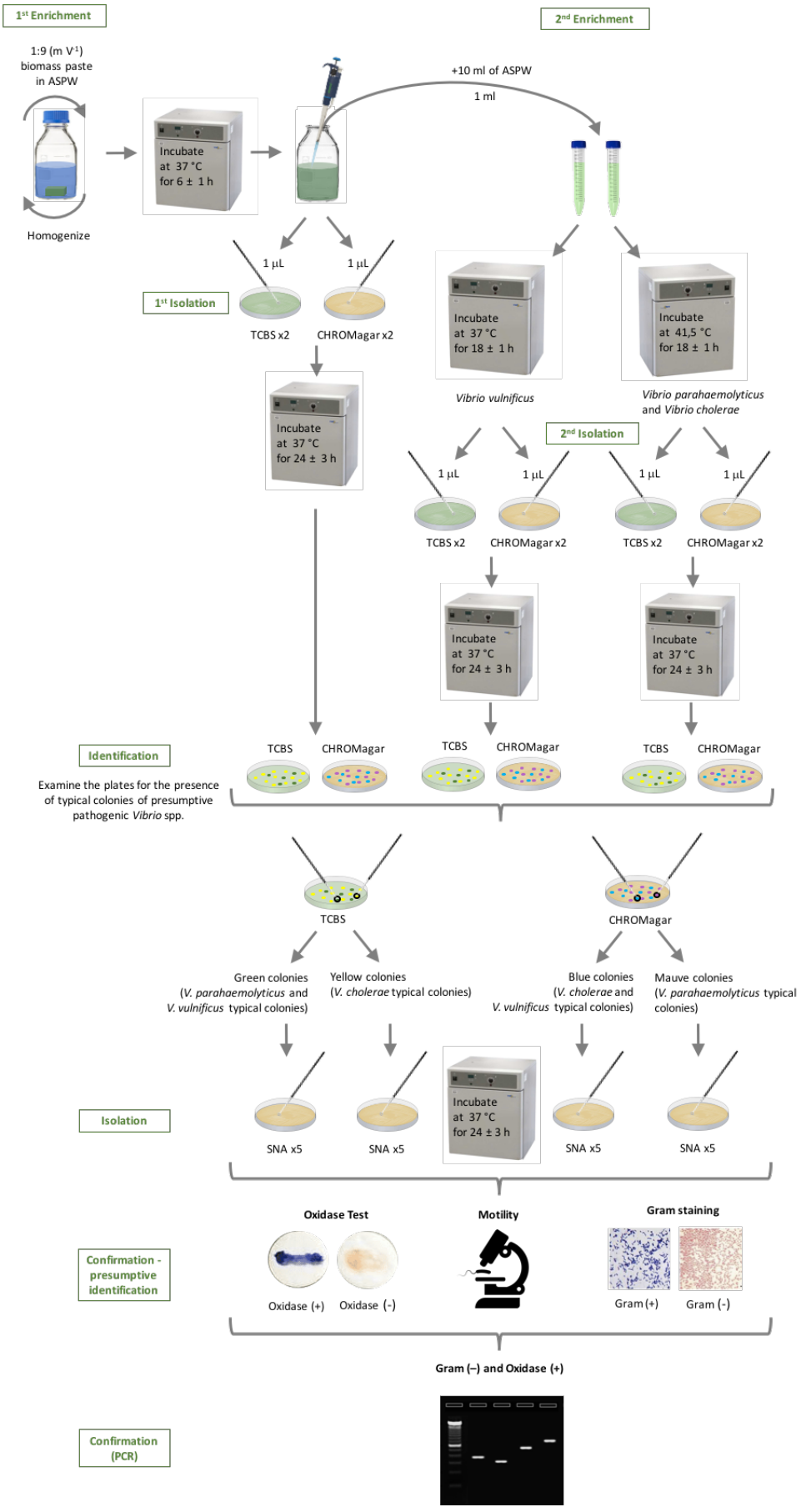


Figure 2.4 - Diagram of the method tested, according to the ISO 21872-1:2017.

2.5.2.5 Confirmation - tests for presumptive identification

To select colonies for confirmation and preparation of pure cultures, from each of the two selective media, five colonies considered to be typical or similar to each of the potentially pathogenic *Vibrio* spp. were subcultured onto the surface of Saline Nutrient Agar (SNA) plates (pH 7.2, containing 5 g meat extract, 3 g peptone, 10 g NaCl, 15 g agar to 1 L of deionized distilled water) and incubated at 37 °C for 24 ± 3 h.

The first stage of the confirmation step comprised three tests for presumptive identification: oxidase test, Gram staining and motility.

A. Oxidase test

Kovac's oxidase reagent (1 %) was set by dissolving 0.1 g of tetramethyl-p-phenylenediamine dihydrochloride into 10 mL of sterile distilled water. A portion of the pure culture from the SNA plates was taken using a sampling loop and streaked onto a filter paper moistened with oxidase reagent. The test detects the presence of cytochrome oxidase, being positive if the redox indicator reduced to mauve, violet or deep purple within 10 s (Kersters & Ley, 1971).

B. Gram staining

For each pure culture, a film for Gram staining was prepared according to ISO 7218. A bacterial film was flame-fixed on a microscope slide prepared from a 18 - 24 h culture. The film was covered with the crystal violet that reacted 1 min. It was gently rinsed with water for a few seconds and the slide was covered with the iodine solution (lugol) that reacted for 1 min. After rinsed with water for a few seconds, a film of ethanol 95 % (V V^{-1}) was gently poured onto the slide until no more violet color was washed out. Next, it was gently rinsed with water to eliminate the ethanol and covered with the solution of safranin for 10 s. Finally, it was gently rinsed with water and the slide was dried. After staining, the Gram reaction was examined under the high-power oil objective of a microscope. Bacterial cells which appeared blue or violet were termed Gram-positive. Those colored dark pink to red were termed Gram-negative (ISO, 7218).

C. Motility

A tube of ASPW was inoculated with the sample to be tested and incubated at 37 °C for 6 h. A drop of this culture was deposited onto a slide, covered with a coverslip and examined for motility under the microscope.

Afterward, the oxidase positive and Gram-negative colonies which gave a positive result in the motility test (if examined) proceeded to further confirmation. While ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 (**Figure 6.1** and **Figure 6.2**) offer merely biochemical tests as a way to confirm *Vibrio* spp. identification, ISO 21872-1:2017 (**Figure 6.3**) allows the use of polymerase chain reaction (PCR) as an alternative to biochemical tests. According to the ISO/TS 21872:2007, the next step was the biochemical tests. Although they were not performed in these preliminary tests, due to laboratory limitations, the detection of potentially enteropathogenic *Vibrio* spp. would be later done according to ISO 21872-1:2017, where the biochemical confirmation step is replaced by PCR and therefore would not offset the investment in being tested.

2.5.2.6 PCR confirmation

Regarding the ISO 21872-1:2017, the PCR was the chosen method for the confirmation step, over the biochemical tests. As shown in **Table 2.3**, the PCR comprised primers targeting the three species *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. ISO 21872-1:2017 also presented two primer sets for the determination of *V. parahaemolyticus* pathogenic strains, targeting the genes encoding the thermostable direct (TDH) and the thermostable direct related (TRH) haemolysins. Although, these primers were not implemented considering that, as previously stated by Raghunath (2015) and later by Bhunia (2018), approximately 10 % of clinical strains do not contain *tdh* and/or *trh.* Even more, environmental isolates of *V. parahaemolyticus* lacking both *tdh* and *trh* are also highly cytotoxic to human gastrointestinal cells, due to the expression of other virulence factors, such as extracellular proteases, biofilm and siderophore (Raghunath, 2015).

Additionally to ISO 21872-1:2017, a primer set targeting the genus *Vibrio* (Kim *et al.,* 2015)*,* was also tested as additional information to the other three primer sets. ISO 21872-1:2017 method does not detect all species of the genus *Vibrio* but merely the three ones specified: *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Henceforth, the information given by the genus *Vibrio* primers is merely additional information and cannot, for itself, replace the ISO 21872-1:2017 and officially conclude the presence or absence of *Vibrio* spp. in the sample.

PCR was also used to screen for the presence of *Vibrio* spp. in the secondary enrichment broths following incubation.

Table 2.3 - Primer sets for PCR confirmation step and expected product sizes for each target. Degenerate primers: R=A+G; Y=C+T; N=A+C+G+T; S=C+G.

Identification	Specific target	Primer	Product size (bp)
V. cholerae	*prVC*	*prVC* (FW): TTA AGC STT TTC RCT GAG AAT G *prVC* (REV): AGT CAC TTA ACC ATA CAA CCC G	295 to 310
V. parahaemolyticus	*toxR*	*VpToxR* (FW): GTC TTC TGA CGC AAT CGT TG *VpToxR* (REV): ATA CGA GTG GTT GCT GTC ATG	368
V. vulnificus	*VVH*	*VVH* (FW): CCG GCG GTA CAG GTT GGC GC *VVH* (REV): CGC CAC CCA CTT TCG GGC C	519
Genus *Vibrio* (Kim *et al.*, 2015)	recombinase A region	raVG (FW): GTC ARA TTG AAA ARC ART TYG GTA AAG G raVG (REV): ACY TTR ATR CGN GTT TCR TTR CC	689

A. DNA extraction

Instead of testing each colony individually for each of the primer sets, doing one DNA extraction for each, five colonies of each type were pulled together into a single DNA extraction. Differently than the ISO 21872:2007, the ISO 21872-1:2017 only requires the confirmation of one colony of each typical morphology and medium. Hence, when this step is implemented along the final method to be performed,

only one colony should be tested. Although, during implementation, in order to have better reliability in the results obtained, to infer if the colonies detected are or not *Vibrio* spp., five colonies were tested.

To prepare a bacterial suspension, five colonies were taken from SNA and inoculated into 200 µL of sterile nuclease free water. The tubes were heated in a thermocycler at 95 ± 2 °C for 5 ± 1 min, centrifuged at 10000 x *g* for 1 min and the supernatant was retained for PCR testing. According to the original ISO 21872-1:2017 method, a heating block was required, although, due to its nonexistence at A4F's laboratory, a thermocycler was used instead. Consequently, the 500 µL of sterile nuclease free water established had to be reduced to 200 µL in order to fit the equipment.

For the PCR directly to the enrichment broth, 200 µL of the broth were heated at 95 ± 2 °C for 5 ± 1 min, centrifuged at 10000 x *g* for 1 min and the supernatant was retained for PCR testing.

B. Master mix and primers

The master mix was prepared according to **Table 2.4** using the appropriate primers (**Table 2.3**) and adjusting volumes depending on the required number of reactions.

Table 2.4 - Composition of master mix.

Reagent	Final concentration
NZYTaq II 2x Green Master Mix	1 x
Forward primer (100 µM)	1.04 µM
Reverse primer (100 µM)	1.04 µM
Ultrapure Water	Add to 24 µL
Total volume	24

Instead of the 48 µL described in the ISO, the total volume was established as 24 µL, keeping the same concentrations. This was possible since only 20 µL were required to load the gel. Even more, this decision allowed to maximize the material available.

C. Conventional PCR

Extracted DNA (1 µL) was added to a clearly labelled tube with 24 µL of master mix. A negative control was performed for each batch of samples tested, consisting on a negative extraction control with nuclease free water instead of extracted DNA. Positive controls were used for each target *Vibrio* species. The PCR tubes were loaded into the thermocycler and the appropriate running conditions were set depending on the primer set used (**Table 2.5**, **Table 2.6** and **Table 2.7**).

Table 2.5 - Cycling parameters - *VptoxR* and *VVH*.

Step description		Temperature and time	Number of cycles
Pre-heating		96 °C for 5 min	1
Amplification	**Denaturation**	94 °C for 1 min	30
	Annealing	63 °C for 1.5 min	
	Extension	72 °C for 1.5 min	
Post amplification		72 °C for 7 min	1

Table 2.6 - Cycling parameters – *prVC*.

Step description		Temperature and time	Number of cycles
Pre-heating		94 °C for 2 min	1
Amplification	**Denaturation**	94 °C for 1 min	30
	Annealing	50 °C for 1 min	
	Extension	72 °C for 1.5 min	
Post amplification		72 °C for 10 min	1

Table 2.7 - Cycling parameters – genus *Vibrio* (Kim *et al*., 2015).

Step description		Temperature and time	Number of cycles
Pre-heating		94 °C for 5 min	1
Amplification	**Denaturation**	94 °C for 30 s	25
	Annealing	60 °C for 30 s	
	Extension	72 °C for 30 s	
Post amplification		72 °C for 10 min	1

An agarose gel (2 %) was prepared by mixing 2 g of agarose with 100 mL of 1X TAE buffer. After cooling down, a few drops of Green Safe Premium were added, enabling visualization of the product. The gel was poured into the mould and let to solidify before removing the comb. Afterwards, the gel was loaded with 10 µL of DNA ladder and the following wells with 20 µL of PCR products prepared previously. Finally, the gel was run at 130 V for 25 min and, following electrophoresis, visualized using an UV transilluminator.

2.6 ISO 21872-1:2017 method - Spiked Samples

The final experiment to implement the ISO 21872-1:2017 method at A4F consisted on testing samples artificially contaminated with *Vibrio* spp. to infer the specificity and sensitivity of the method and if the method can detect its presence or if the false positives mask the true positives leading to false conclusions (FDA, 2015). With this aim in mind, *Nannochloropsis* sp. paste, previously tested as negative for *Vibrio* spp. by a reference laboratory, was analyzed as a negative control and two samples from the same paste were artificially contaminated with approximately 10 and 100 CFU of each species of *Vibrio*.

2.6.1 Spiked samples preparation

As shown in **Figure 2.5** a single colony of each of the three *Vibrio* spp. was separately inoculated into 5 mL of LB pH 8. The culture was incubated in an orbital incubator overnight at 37 °C with vigorous

shaking, 200 rpm. Considering that an overnight culture reaches 10^9 CFU mL^{-1} (Martinez *et al.*, 2010), a sample was collected, and serial dilutions up to 10^{-7} were prepared. From the dilutions 10^{-7} and 10^{-6}, 100 µL were inoculated into the glass bottles comprising already 25 g of paste and 225 mL of ASPW. This inoculum theoretically corresponded to approximately 10 and 100 CFU, respectively, Afterward, 100 µL of the dilutions 10^{-4} - 10^{-7} were inoculated in LB plates (triplicates), and after 18 h of incubation at 37 °C, CFU were counted on the plates presenting between 30 and 300 colonies (Maier *et al.*, 2000).

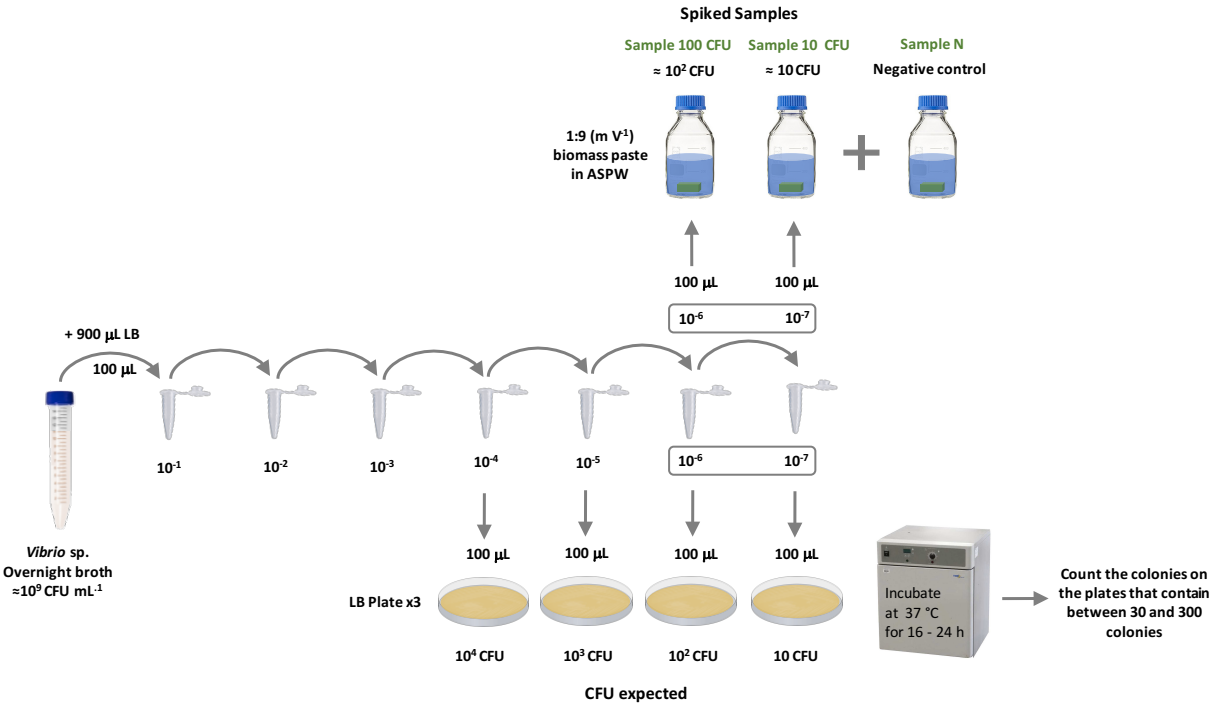


Figure 2.5 - Preparation of *Vibrio* spp. inoculum for spiked samples and CFU counts.

2.6.2 Sample Analysis

The three samples: negative control (sample N), sample inoculated with ≈ 10 CFU (sample ≈ 10 CFU) and sample inoculated with ≈ 100 CFU (sample ≈ 100 CFU) were tested individually. **Figure 2.6** presents the method adapted from ISO 21872-1:2017 for the sample analysis. The main modification was the fact that the Gram staining and oxidase tests were omitted and the colonies obtained on TCBS and CHROMagar™ proceeded directly to PCR.

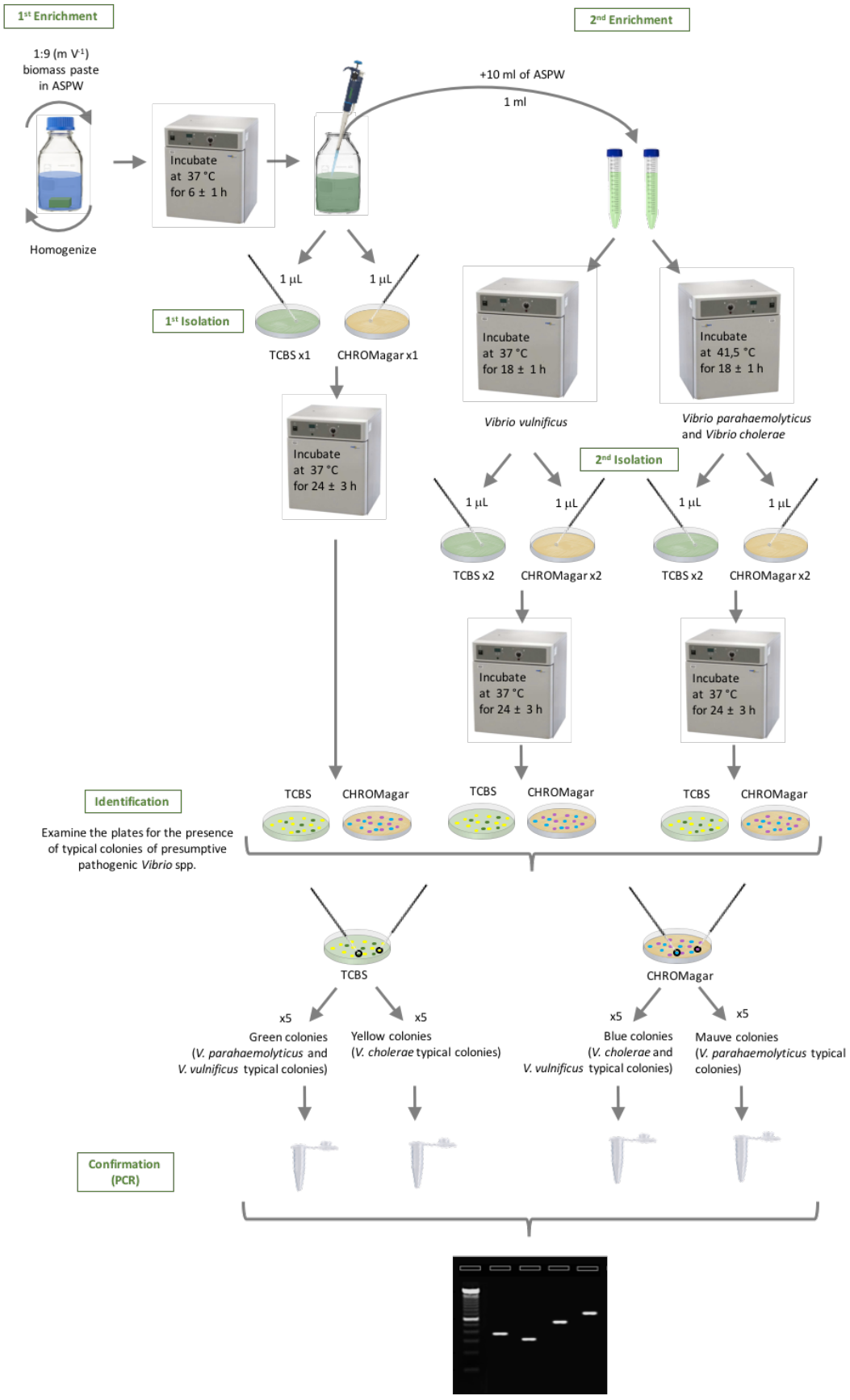


Figure 2.6 - Diagram of the method applied, according to the ISO 21872-1:2017 for the analysis of the spiked samples.

Chapter 3 - Results and Discussion

3.1 Correlation between OD and CFU mL^{-1}

A *V. parahaemolyticus*'s growth curve in LB, pH 8, incubated at 37 °C with shaking (200 rpm), presenting an initial $OD_{600\ nm}$ = 0.056 is showed in **Figure 3.1**. The curve presented an extremely brief *lag* phase followed by exponential and stationary phase. This curve is in accordance with the usual microbial growth curves (Martinez *et al.*, 2010). When inoculated into fresh media, growth begins only after an interval called *lag* phase because the cells are drained of numerous vital components and it is required time for their biosynthesis. Although, if an exponentially growing culture is transferred under the same conditions of growth and medium, there is no *lag* phase and exponential growth begins immediately. In the exponential phase the number of cells doubles in a constant time interval. In stationary phase growth becomes limited mainly due to the fact that an essential nutrient in the culture medium is depleted or the organism's waste products accumulate. The growth rate equals the death rate and the OD remains constant, as seen on the growth curve obtained. Eventually, the population enters the death phase of the growth cycle. Nevertheless, in this experiment, the culture did not grow long enough to reach this last stage (Madigan *et al.*, 2015). Comparing to other growth curves for *Vibrio* spp. in literature, the maximum value of OD obtained is smaller which can be explained by the fact that the container used was a T flash. Possibly, the 200 rpm shaking applied was excessive for this type of container, comparing to an Erlenmeyer commonly used for bacteria cultivation (Martinez *et al.*, 2010).

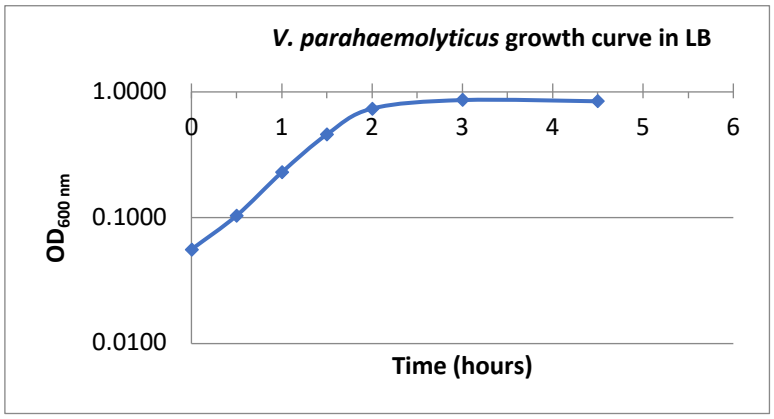


Figure 3.1 - *V. parahaemolyticus* growth curve in LB in logarithmic scale.

Table 3.1 compiles the values obtained for the correlation between OD and CFU mL^{-1} in order to determine the limit of detection of FISH. Beyond the dilutions corresponding to the growth times 0.5 h, 1 h and 1.5 h, the 2 h long culture was also plated but the latest presented values that were not congruent with the first three, showing a higher OD but lower CFU counts. This inconsistency can be the result that some of the cells, already in stationary phase, were not viable, which resulted in a lower number of CFU. Considering this fact, CFU count with the sample incubated for two hours were not included in the correlation **(Figure 3.2)**. The equation obtained for the correlation, $y = 10^9 x + 7 \cdot 10^8$ corresponds to $CFU = (10^9) \cdot (OD) + 7 \cdot 10^8$. The correlation between OD and CFU mL^{-1} was merely performed for *V. parahaemolyticus,* and not for *V. vulnificus* and *V. cholerae* due to limitations on the biological safety cabinet availability. Even more, this extrapolation provided enough information for the company main goal.

Table 3.1 - Values obtained for the correlation between OD measured at 600 nm and CFU mL^{-1}.

Time (h)	OD	CFU counts (Triplicates)	CFU mL^{-1} (in the Original Suspension)
0	0.0560	-	-
0.5	0.1040	86; 78; 70	$7.00 \cdot 10^8$
1	0.2293	90; 98; 89	$9.00 \cdot 10^8$
1.5	0.4595	119; 113; 114	$1.14 \cdot 10^9$
2	0.7373	112; 102; 105	$1.05 \cdot 10^9$
3	0.8613	-	-
4.5	0.8423	-	-

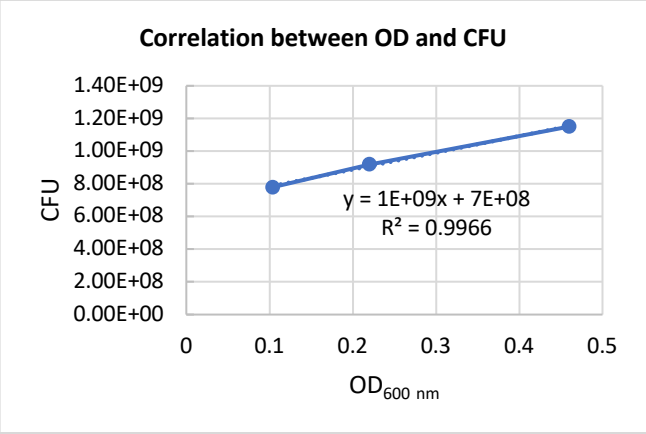


Figure 3.2 - Correlation between OD measured at 600 nm and CFU mL^{-1}.

3.2 FISH - hybridization procedure on suspension

Figure 3.3 shows the positive controls for *V. cholerae* (A) and *V. vulnificus* (B) after overnight growth in LB, pH 8, incubated at 37 °C with shaking (200 rpm).

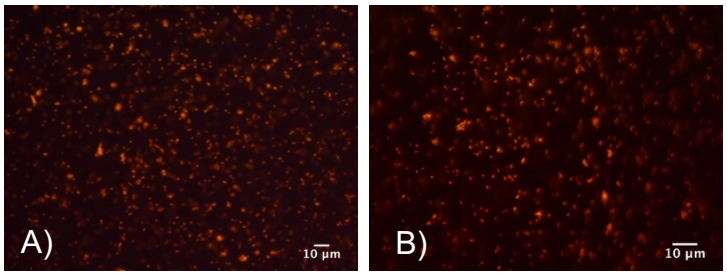


Figure 3.3 - Fluorescence microscopic observation of *V. cholerae* (A) and *V. vulnificus* (B) positive controls with each species' specific probe, after growth in LB overnight. FISH signals represent a specific sequence of 16S rRNA (red).

3.2.1 FISH - Hybridization procedure on suspension including autofluorescence inhibition

Algae exhibit strong autofluorescence from its photosynthetic pigments, which emission is dependent on its metabolic activities and physiological state. In **Figure 3.4**, a sample of *Nannochloropsis* from a cultivation in a photobioreactor can be observed under phase contrast and fluorescence microscopy. As noticeable, one of the problems faced with FISH technique is the autofluorescence of the microalgae, which masks the fluorescence of the fluorophore Cy3, using the most suitable filter cube for it.

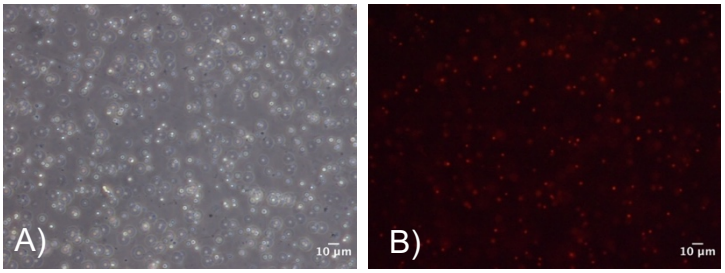


Figure 3.4 - *Nannochloropsis* culture under phase contrast (A) and fluorescence (B) microscopy.

The *Nannochloropsis* sample was treated with Sudan Black B to test the ability of this stain to inhibit the autofluorescence. **Figure 3.5** presents the results obtained, where we can see that Sudan Black B was able to inhibit the autofluorescence.

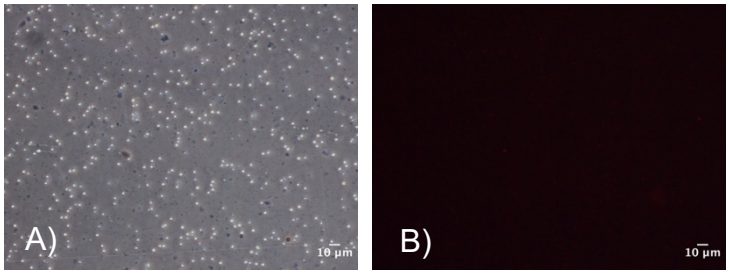


Figure 3.5 - *Nannochloropsis* culture after treatment with Sudan Black B, under phase contrast (A) and fluorescence (B) microscopy.

3.2.2 Determination of the detection limit

The determination of the detection limit of the FISH method was achieved using *V. parahaemolyticus*. The results obtained are presented below. The negative controls for each sample were not included, consisting merely of similar pictures but with no fluorescence. Regarding the control to confirm the absence of *V. parahaemolyticus* in the original *Nannochloropsis* culture (**Figure 3.6**), *V. parahaemolyticus* was not detectable through FISH. **Figure 3.7** shows the positive results, as expected, for *V. parahaemolyticus* control (10^9 CFU mL^{-1} according to the correlation presented in **Figure 3.2)** with the probe, treated with Sudan Black B. The following pictures present the samples of *Nannochloropsis* cultures artificially contaminated with serial dilutions of *V. parahaemolyticus*, treated with Sudan Black B (**Figure 3.8**, **Figure 3.9**, **Figure 3.10** and **Figure 3.11**). In concentrations below 10^6 - 10^5 CFU mL^{-1}, *V. parahaemolyticus* was not detected. Hence, the limit of detection of the FISH method with Sudan Black B to inhibit the autofluorescence of the microalgae is 10^6 - 10^5 CFU mL^{-1}. Although, at 10^5 CFU mL^{-1} it was challenging to find *V. parahaemolyticus* along the sample. Hence, the trustable limit of detection considered should be 10^6 CFU mL^{-1}. The limit of detection obtained is within the value expected for microscopic observation (Biomode SA, n.d). Even though the limit of detection would be improved if the method was done with flow cytometry, the fact that Sudan Black B was added obstructed this option, since it could interfere with the results obtained. Therefore, if the microalgae culture is contaminated with *Vibrio* sp. with a concentration lower than 10^6 CFU mL^{-1}, the method is not be able to detect it. Firstly it should be taken into consideration that *Vibrio* spp. have never been detected in microalgae cultures at A4F. In previous studies, the addition of *Nannochloropsis* to rearing water tanks suppressed both quantitatively and qualitatively the growth of some *Vibrio* spp. (Taniguchi *et al.*, 2011). This is a possible explanation for the absence of these *Vibrio* spp. in the cultures. Considering this fact, even if present, the CFU mL^{-1} would be expected to remain low, close to zero CFU mL^{-1} of

culture. This way, considering the detection limit of the method, an enrichment step, such as enrichment in ASPW would be required in order to evaluate only the presence of *Vibrio* spp.

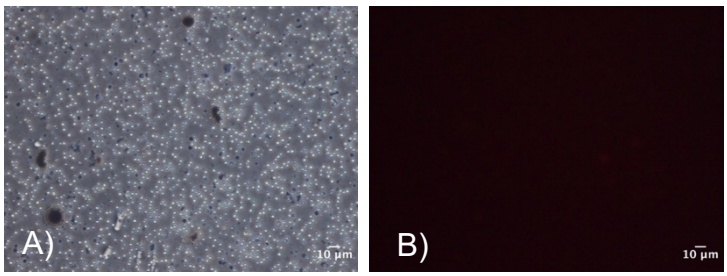


Figure 3.6 - *Nannochloropsis* culture after treatment with Sudan Black B, with the species' specific probe under phase contrast (A) and fluorescence (B) microscopy.

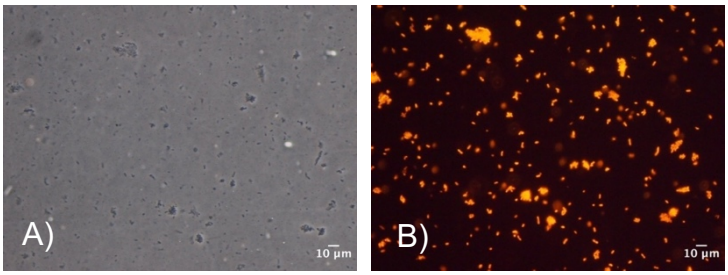


Figure 3.7 - Fluorescence microscopic observation of *V. parahaemolyticus* positive control (10^9 CFU mL^{-1}) with the species' specific probe, treated with Sudan Black B. Under phase contrast (A) and fluorescence (B) microscopy.

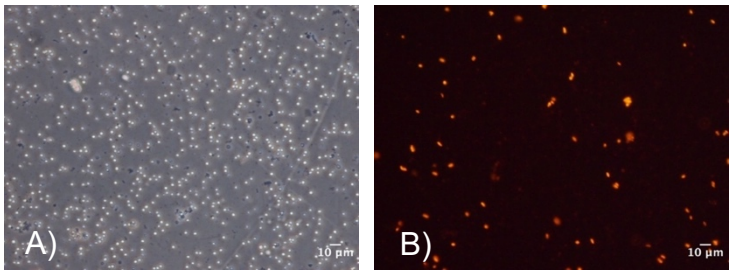


Figure 3.8 - *Nannochloropsis* culture sample artificially contaminated with 10^8 CFU mL^{-1} of *V. parahaemolyticus*, treated with Sudan Black B. Under (A) and fluorescence (B) microscopy.

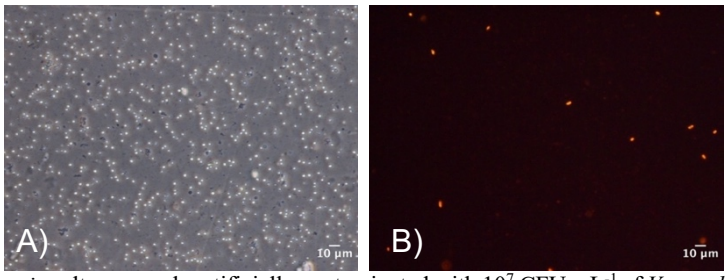


Figure 3.9 - *Nannochloropsis* culture sample artificially contaminated with 10^7 CFU mL^{-1} of *V. parahaemolyticus*, treated with Sudan Black B. Under phase contrast (A) and fluorescence (B) microscopy.

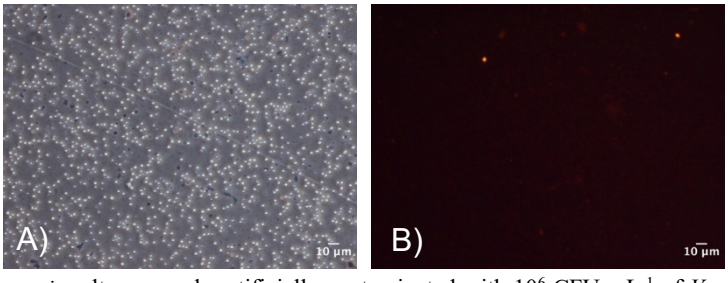


Figure 3.10 - *Nannochloropsis* culture sample artificially contaminated with 10^6 CFU mL^{-1} of *V. parahaemolyticus*, treated with Sudan Black B. Under phase contrast (A) and fluorescence (B) microscopy.

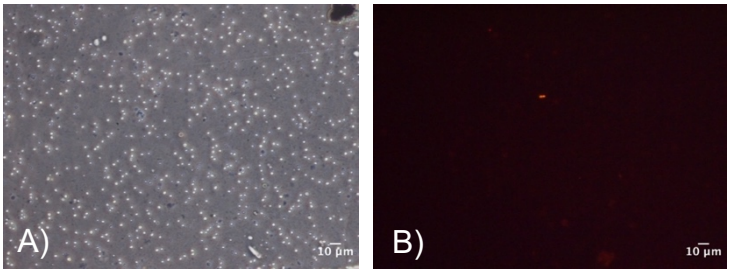


Figure 3.11 - *Nannochloropsis* culture sample artificially contaminated with 10^5 CFU mL^{-1} of *V. parahaemolyticus*, treated with Sudan Black B. Under phase contrast (A) and fluorescence (B) microscopy.

3.3 Standard Method

3.3.1 Preliminary tests

Since it was not possible to promptly acquire the ISO 21872-1:2017 dedicated to the detection of *V. parahaemolyticus, V. cholerae* and *V. vulnificus*, these preliminary tests were performed according to both parts of the ISO/TS 21872:2007. The purpose of these experiments was not to implement the full method but to infer the efficiency of the selective enrichment and the TCBS to detect false positives, consequently affecting the steps required until the absence of *Vibrio* spp. in samples, previously tested as negative, could be concluded.

3.3.1.1 Positive Controls

Beyond confirming the typical morphology of the positive controls' colonies, this step aimed to confirm the efficiency of the sample's first enrichment. To do so, in parallel to the sample analysis, the same method was applied to the positive controls. **Figure 3.12** and **Figure 3.13** respectively present the TCBS plates from the positive controls inoculated with the 1:100 dilution in medium used for the microalgae cultivation and the following 1:10 dilution in ASPW, simulating the sample's first enrichment. Regarding the efficiency of the enrichment, when analyzing the positive controls plates before (**Figure 3.12**) and after (**Figure 3.13**) the first enrichment, even though a 1:10 dilution was performed, a higher number of colonies was observed afterward. Henceforth, the enrichment worked efficiently. For both cases, while *V. cholerae* grew to form smooth, yellow colonies with 2 - 3 mm in diameter, *V. parahaemolyticus* grew as smooth, green colonies with 2 - 3 mm in diameter. Both results are concordant to the description of typical colonies given by ISO/TS 21872:2007. Contrarily, *V. vulnificus* does not match the description of typical colonies of this species since it formed smooth, yellow colonies with near 2 mm in diameter rather than the smooth, green colonies with 2 - 3 mm in diameter expected. Although, different studies stated that some *V. vulnificus* can, in fact, ferment sucrose resulting in yellow colonies instead of the green colonies expected (Kim *et al.*, 2010; Nagao *et al.*, 2006). With this step, it is possible to infer that sucrose fermentation is not such a discriminatory character to differentiate *Vibrio* spp. Even though, since the goal of the analysis is to detect the presence of all *Vibrio* spp. covered by the ISO, all green or yellow colonies similar to *Vibrio* spp. proceed to further confirmation steps. Hence, this setback does not influence the method. As seen in **Figure 3.13B)** and **Figure 3.13C)**, these plates presented an almost confluent growth on the entire plate, with isolated colonies only on the borders. This experiment also point out that a better inoculum distribution through the plate must be achieved in the future assays.

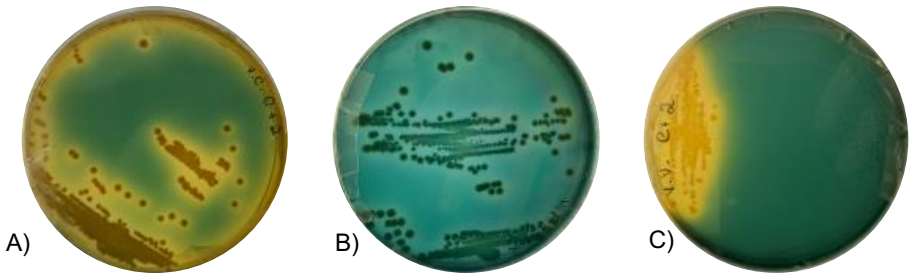


Figure 3.12 - TCBS plates inoculated with the 1:100 dilution of *V. cholerae* (A), *V. parahaemolyticus* (B) *and V. vulnificus* (C) in medium used for the microalgae cultivation.

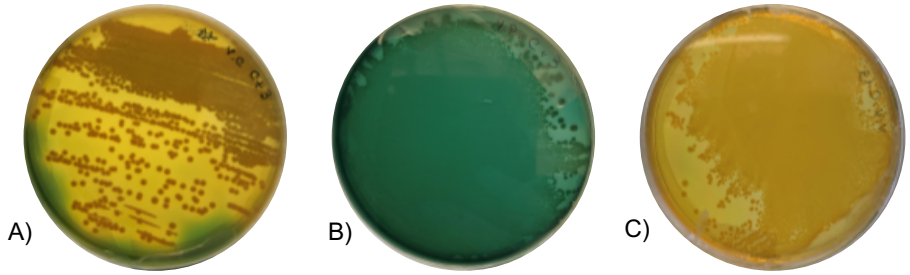


Figure 3.13 - TCBS plates inoculated with the 1:10 dilution of *V. cholerae* (A), *V. parahaemolyticus* (B) *and V. vulnificus* (C) in ASPW after the first enrichment.

3.3.1.2 Detection of *Vibrio* spp. in the sample

The plates inoculated with the first enrichment (**Figure 3.14**) presented two smooth, yellow colonies with 2 - 3 mm in diameter on plate A) and one colony on plate B).

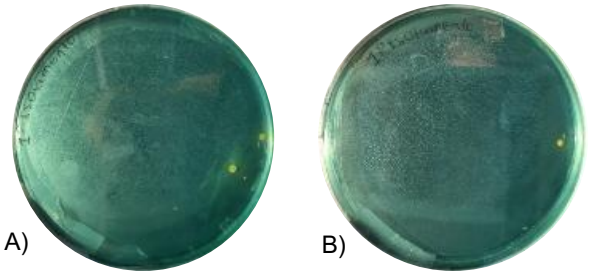


Figure 3.14 - Duplicates of TCBS plates (A) and (B) inoculated with the first enrichment incubated at 37 °C.

The plates inoculated with the second enrichment incubated at 37 °C and 41.5 °C (**Figure 3.15)** showed two types of colonies. The yellow smooth colonies and the black smooth colonies had approximately 1 mm in diameter. No differences were identified between the plates inoculated with the second enrichment incubated at 37 °C or 41.5 °C. Additionally, when these plates were manipulated, a sulfur smell was noticed.

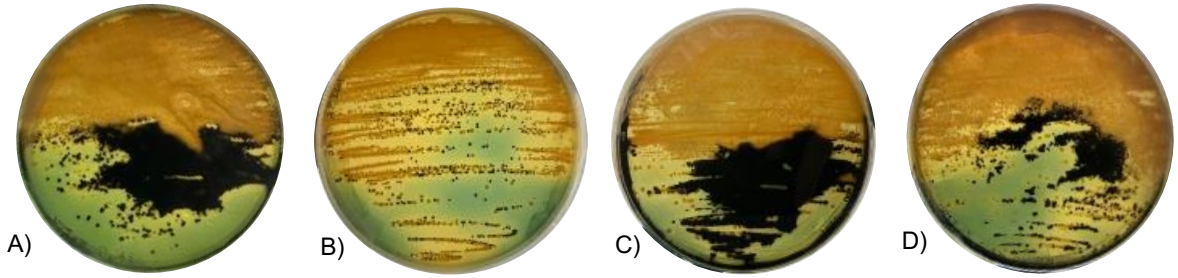


Figure 3.15 - Duplicates of TCBS plates inoculated with the second enrichment incubated at 37 °C (A) and (B) and at 41.5 °C (C) and (D).

Regarding the black colonies, the sodium thiosulfate on TCBS serves as a source of sulfur, allowing the production of hydrogen sulphide (H_2S) detected by the ferric citrate, originating black precipitates (Corry *et al*., 2003). Since *Vibrio* spp. do not produce H_2S, these black colonies can be ruled out as vibrio. Considering that these colonies grew under aerobic conditions when plated, they do not represent typical sulfate-reducing microorganisms, which are strict anaerobic (Muyzer & Stams, 2008). Even though, the ability to reduce sulfur is also spread to facultative anaerobic bacteria, such as those belonging to the genus *Proteus* and *Salmonella,* that also exhibit the ability to reduce sulfur compounds such as thiosulfate (Clarke, 1953).

Concerning the yellow colonies detected, they were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. cholerae* or *V. vulnificus* according to the control obtained during this study. Even if the size of the colonies detected was relatively smaller than expected, this difference range, for itself, cannot eliminate them as presumptive *Vibrio* spp. since the *V. vulnificus* positive control also presented the same colonies size. Hence, five of these colonies were subcultured on SNA and proceeded to the confirmation step.

For future assays, it is important to consider that *Vibrio* spp. are facultative anaerobic and grow best where abundant oxygen is present (Chart, 2002). Hence, even though the sample was taken from the surface of the tube, the tubes were homogenized during the final stage of incubation. In future assays, regardless sedimentation, the tubes should be kept steady and when transferring culture or inoculating plates it should always be taken from the surface. This results in an improved *Vibrio* spp. detection as they are mostly present at the surface, where there is abundant oxygen.

The **confirmation step (tests for presumptive identification)** included Gram staining, oxidase test and examination of motility. Regarding Gram staining, all the yellow colonies on TCBS presented a pink to red color, considered Gram-negative microorganisms like *Vibrio* spp. Concerning the oxidase test, like *Vibrio* spp. positive controls, all the isolates were oxidase positive, since the redox indicator was reduced to purple, detecting cytochrome oxidase. Finally, according to the ISO/TS 21872:2007, all *Vibrio* spp. should be motile. The microscopic observations for the examination of motility showed that *V. cholerae* cells were highly motile. Although, contrarily to expectations, *V. parahaemolyticus* presented only few motile cells whereas none was observed with movement for *V. vulnificus* control. Regarding the isolates obtained from the sample, some showed absence of motility while for others few cells with motility were detected. Since two of the three controls also showed almost lack of motility, contrarily to what is mentioned in ISO/TS 21872:2007, the results obtained on the motility test are not that accurate. Even more, the fact that contrast phase was only available at 40 x amplification made this task arduous. Considering these facts, absence of motility should not discard isolates as possible *Vibrio* spp. and, due to its non-obligation, this test was no longer performed. The inability to identify the false positives with Gram staining, oxidase test and observation for motility leads to the requirement of further confirmation steps either in the form of biochemical tests or as PCR according to ISO 21872-1:2017.

With these preliminary tests, we can infer that the selective enrichment step followed by plating on TCBS is not as selective as stated by manufacturers. Considering that the sample was previously tested as negative for *Vibrio* spp. by a reference laboratory, TCBS allows the growth of microorganisms other than the targeted *Vibrio* spp., which results in false positives with 1 mm diameter colonies. Even though false positives on TCBS are still expected, with the following improvements such as taking samples from the surface, it is expected a decrement of false positives which could influence the number of steps required in the analysis.

3.3.2 ISO 21872-1:2017 method

The next step was the application of the current reference method for the detection of potentially enteropathogenic *Vibrio* spp., the ISO 21872-1:2017. This ISO comprises the main novelty that the confirmation step can be performed by PCR as an alternative to biochemical tests and it clearly states that samples should always be taken from the surface. ISO 21872-1:2017 also recommends the use of a chromogenic selective media as the second selective media. As previously mentioned, a variety of chromogenic media were developed to detect *Vibrio* spp. such as Vibrio ChromoSelect® Agar (Sigma-Aldrich), CHROMagar™ Vibrio (CHROMagar™) and ChromID® Vibrio (BioMérieux). From these options, Vibrio ChromoSelect® Agar was excluded due to the fact that it merely differentiated *V. cholerae* and *V. parahaemolyticus*, not mentioning *V. vulnificus* (Sigma-Aldrich, n.d). Then, the choice came to CHROMagar™ Vibrio *versus* ChromID® Vibrio since both were developed for *V. cholerae, V. parahaemolyticus* and *V. vulnificus* detection. Although it has not been an extensive evaluation of the performance of these media, both media were stated to be more effective than TCBS (BioMérieux, n.d.; CHROMagar™, n.d.). Finally, considering A4F's suppliers and the cost of each medium, CHROMagar™ was selected as the second selective medium.

With this experiment, the specific goals were to test if there would be a reduction in the number of false positive colonies with samples being taken from the surface and to infer the efficiency of CHROMagar™ as the second selective differential medium. Another important goal was to test PCR method as the confirmation step. Even more, the PCR step would allow to confirm the positive controls, especially *V. vulnificus*. An additional goal was to compare the results obtained with direct PCR to the enrichment broths, supplementary to the ISO 21872-1:2017 method, to the results obtained with the official method.

3.3.2.1 Positive Controls

Figure 3.16 and **Figure 3.17** respectively present the TCBS plates from the positive controls inoculated with the 1:100 dilution in medium used for the microalgae cultivation and the following 1:10 dilution using ASPW, simulating the sample's first enrichment. While *V. cholerae* grew to form smooth, yellow colonies with less than 1 mm in diameter, *V. parahaemolyticus* grew as smooth, green colonies with 2 - 3 mm in diameter. Besides the fact that *V. cholerae* colonies were smaller than expected, both results are concordant to the description of typical colonies given by ISO 21872-1:2017 in terms of sucrose fermentation. Like in the previous experiment, *V. vulnificus* did not match the ISO 21872-1:2017 description of typical colonies for this species since it formed smooth, yellow colonies with around 2 - 3 mm in diameter rather than the smooth, green colonies with 2 - 3 mm in diameter expected.

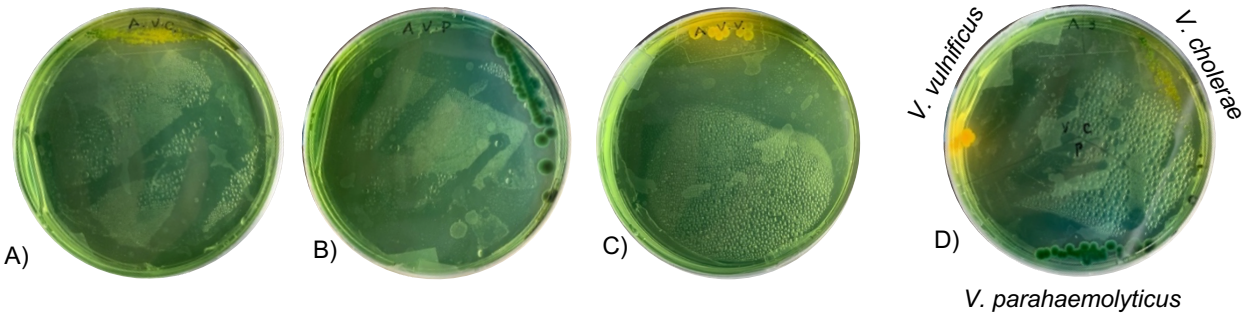


Figure 3.16 - TCBS plates inoculated with the 1:100 dilution in medium used for the microalgae cultivation of *V. cholerae* (A), *V. parahaemolyticus* (B) and *V. vulnificus* (C) and the three species on the same plate (D).

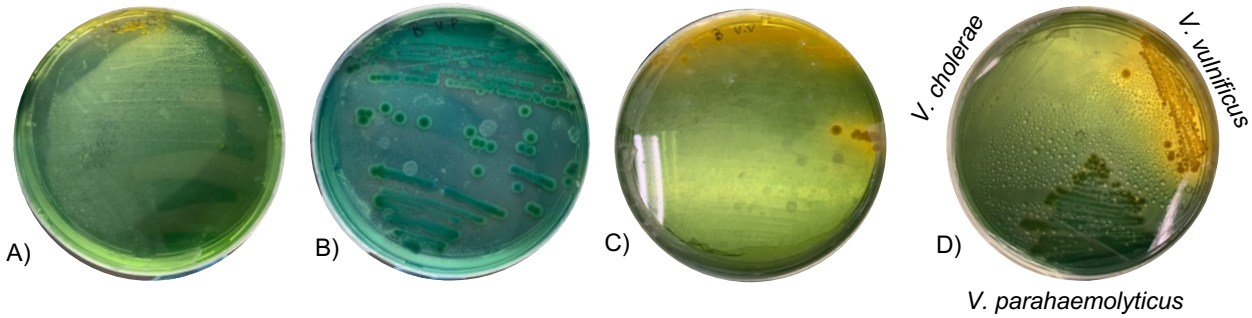


Figure 3.17 - TCBS plates inoculated with the 1:10 dilution in ASPW of *V. cholerae* (A), *V. parahaemolyticus* (B)*, V. vulnificus* (C) and the three species on the same plate (D).

Figure 3.18 presents the CHROMagar™ plates from the positive controls inoculated with the 1:100 dilution in medium used for the microalgae cultivation and the further 1:10 dilution using ASPW. For both cases, while *V. cholerae* and *V. vulnificus* grew to form smooth, turquoise blue colonies with 1 mm in diameter, *V. parahaemolyticus* grew as smooth, mauve colonies with 2 - 3 mm in diameter. The results are concordant to the description of typical colonies given by CHROMagar™ that only presents the expected colony color for each species, disregarding their size (CHROMagar™, n.d.). When analyzing the positive controls plates before (**Figure 3.16** and **Figure 3.18A**) and after the first enrichment (**Figure 3.17** and **Figure 3.18B**) it is possible to infer that the enrichment worked efficiently.

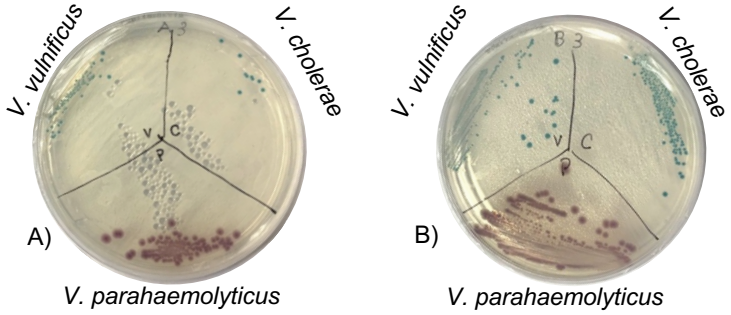


Figure 3.18 - CHROMagar™ plates inoculated with the three species 1:100 dilution in medium used for the microalgae cultivation (A) and 1:10 dilution in ASPW (B).

3.3.2.2 Detection of *Vibrio* spp. in the sample

The **first isolation** plates showed no colonies either on TCBS or CHROMagar™. Differently, the **second isolation** TCBS plates inoculated with the second enrichment incubated at 37 °C (**Figure 3.19A** and **Figure 3.19B**) and 41.5 °C (**Figure 3.20A** and **Figure 3.20B**) presented one type of colonies, yellow smooth colonies with less than 1 mm in diameter. No differences were observed between the plates inoculated with the second enrichment incubated at 37 °C or 41.5 °C. On CHROMagar™, the plates inoculated with the second enrichment incubated at 37 °C (**Figure 3.19C** and **Figure 3.19D**) and at 41.5 °C (**Figure 3.20C** and **Figure 3.20D**) presented differences. At 37 °C three types of colonies were detected. The majority of the colonies were colorless and an agglomerate of mauve and turquoise blue colonies was obtained, with no isolated colonies. Contrarily, at 41.5 °C only colorless colonies were present. This difference between the colonies obtained at the two temperatures suggests that the optimum growth temperature for these contaminants is at 37 °C rather than 41.5 °C.

Concerning the yellow colonies detected in the second isolation on TCBS plates, although they did not present 2 - 3 mm in diameter, they were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. cholerae* or *V. vulnificus* according to the color of the controls obtained during

this experiment. Hence, five of these colonies were subcultured on SNA and proceeded to the confirmation step. Regarding the colonies detected in the second isolation on CHROMagar™, the colorless colonies were discarded as *V. cholerae, V. vulnificus* or *V. parahaemolyticus.* However, these colonies could correspond to *V. alginolyticus*, another potentially pathogenic *Vibrio* spp. (Mustapha *et al.*, 2013). Consequently, additionally to the ISO 21872-1:2017 method, one colony was isolated on SNA and retained for additional PCR confirmation for the genus *Vibrio*. The mauve and turquoise blue colonies presenting confluent growth on the top of the plate were considered presumptive *Vibrio* spp. due to their similarity with the typical color of colonies of *V. parahaemolyticus* and *V. vulnificus*/*V. cholerae*, respectively. To obtain isolated colonies for further confirmation, a portion of the agglomerate with mauve and blue colonies was taken and inoculated on a new CHROMagar™ plate. After incubation at 37 °C for 24 h, the colonies obtained were smaller than 0.1 mm, while after incubation for further 24 h, isolated colonies with approximately 1 mm were obtained. The range difference between the size of the colonies after 24 h of incubation and the positive controls could, for itself, indicate that these colonies are not *Vibrio* spp. Although, to confirm the method, they still proceeded to confirmation. After incubation at 37 °C for 24 h, the blue colonies originated colonies on SNA. Contrarily, the mauve colonies did not grow on SNA so they proceeded directly to PCR.

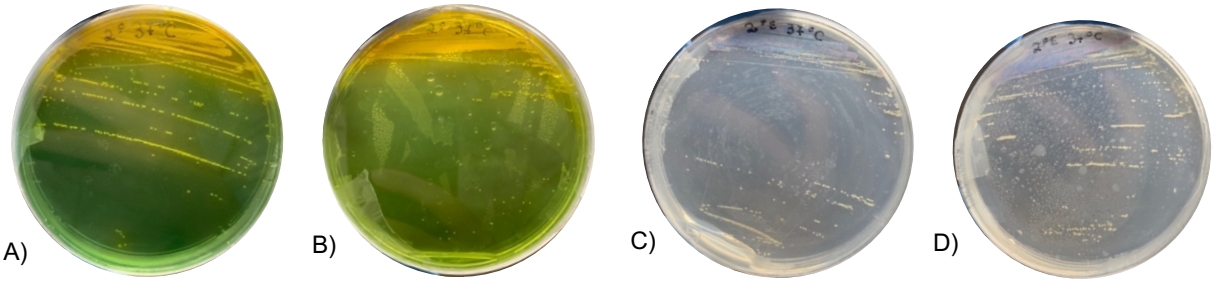


Figure 3.19 - Duplicates of TCBS plates (A) and (B) and of CHROMagar™ plates (C) and (D) inoculated with the second enrichment incubated at 37 °C.

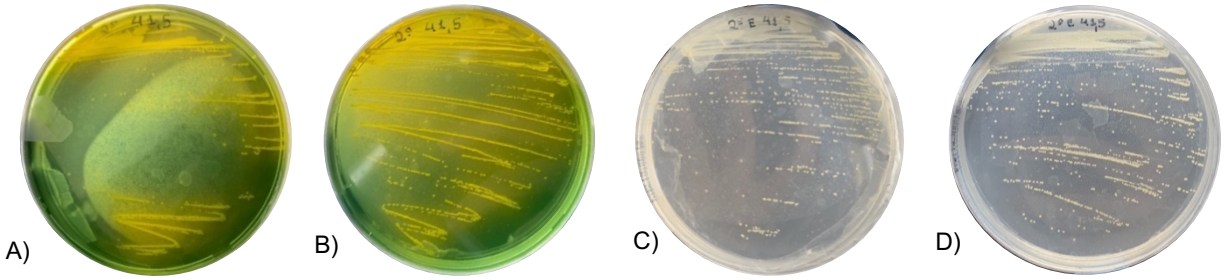


Figure 3.20 - Duplicates of TCBS plates (A) and (B) and of CHROMagar™ plates (C) and (D) inoculated with the second enrichment incubated at 41.5 °C.

Regarding the **confirmation step (tests for presumptive identification),** the yellow colonies on TCBS and blue colonies on CHROMagar™, presented a pink to red color for Gram staining, concluding they are all Gram-negative like *Vibrio* spp. (**Figure 3.21).** Regarding the oxidase test (**Figure 3.22),** like *Vibrio* spp. positive controls, yellow colonies on TCBS presented a positive result detecting cytochrome oxidase since the redox indicator was reduced to purple. Contrarily, blue colonies on CHROMagar™ gave a negative result for oxidase, remaining colorless. Hence, the results obtained in these two tests did not allow the discrimination of the false positives obtained on TCBS but allowed to rule out the blue colonies on CHROMagar™ as *Vibrio* spp.

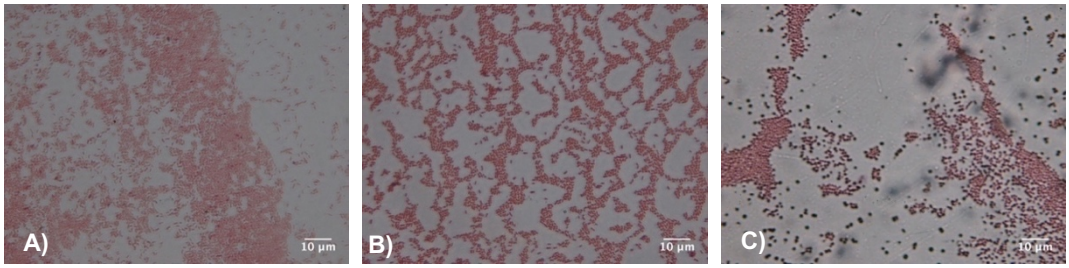


Figure 3.21 - Gram staining results for *V. cholerae* Gram-negative control (A), one isolate from TCBS (B) and one isolate from the blue colonies on CHROMagar™ (C).

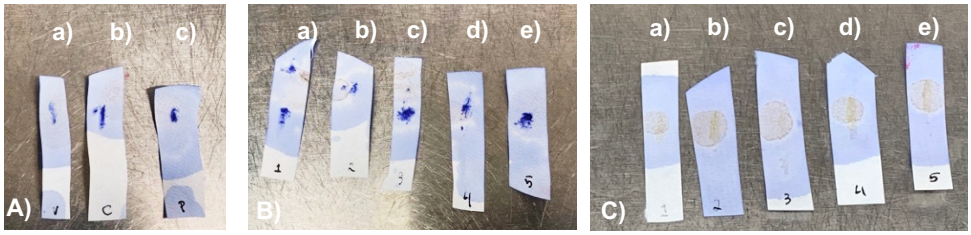


Figure 3.22 - Oxidase test results for the oxidase positive controls (A) *V. vulnificus* (a), *V. parahaemolyticus* (b) and *V. cholerae* (c) and yellow colonies detected on TCBS plates (B) five isolates (a-e) and the blue colonies detected on CHROMagar™ plates (C) five isolates (a-e).

The next step was the **confirmation by PCR.** When comparing the results with the expected product sizes presented in **Table 2.3,** considering the ladder (**Figure 3.23A**), the product sizes for the positive controls obtained in the electrophoresis gels (**Figure 3.24**; **Figure 3.25** and **Figure 3.26**) are in accordance with the expected ones. Hence, we can infer that, even though *V. vulnificus* gave different results in sucrose fermentation than the majority of the strains from this species, by VVH detection this positive control represents *V. vulnificus.*

In addition to the ISO 21872-1:2017 method, PCR was used to screen for the presence of *Vibrio* spp. in the second enrichment broth at 37 °C, following incubation. Considering the resources available, only the enrichment broth at 37 °C was screened since all of the three species were able to grow at 37 °C. The first electrophoresis attempt was run with 1 % agarose gel. Although, as noticeable in **Figure 3.23**, with the voltage and run time applied the samples partially ran out of the gel, it was still possible to infer that there was no amplification for *V. parahaemolyticus, V. cholerae* or *V. vulnificus,* concluding these three species were not present in the second enrichment broth at 37 °C. Additionally, even if extremely faded, a band was detected for the genus *Vibrio*, evidencing the presence of contaminants belonging to this genus, other than the three species targeted individually. **Figure 3.24** presents the same samples after electrophoresis with 2 % agarose. The samples for the genus *Vibrio* did not amplify either for the positive control or the second enrichment sample. This occurrence can be explained by the fact that, unlike on the other PCRs, these reactions were prepared as single and thereby, extremely small volumes were pipetted which could be the cause of insufficient primer concentration in the master mix and therefore, nonappearance of the band corresponding to the genus *Vibrio*. Consequently, in the next assays, a master mix for all the reactions together was always prepared. Additionally, considering that even on the positive controls the genus *Vibrio* band was always faded (**Figure 3.23**; **Figure 3.25** and **Figure 3.26**), a higher concentration of primers should be used or the number of cycles should be increased from 25 to 30 cycles since previous studies stated that 30 cycles resulted in a higher number of copies, ensuring an improved limit of detection (Kim *et al.,* 2015).

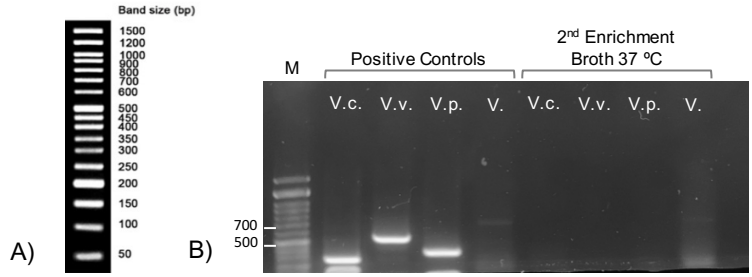


Figure 3.23 - **(A)** Molecular weight size marker - NZY DNA Ladder VI; **(B)** electrophoresis gel (1 % agarose) for the PCR products of the second enrichment broth at 37 °C. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p**.): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

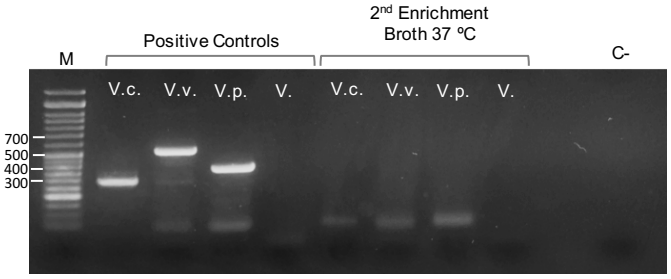


Figure 3.24 - Electrophoresis gel (2 % agarose) for the PCR products of the second enrichment broth at 37 °C. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*; (**C-**): negative control. The primers target is written in white and the sample in test is indicated in black.

The oxidase positive and the Gram-negative colonies detected on TCBS and CHROMagar™ plates were considered presumptive *Vibrio* spp. and retained for further confirmation with PCR. Hence, the yellow colonies on TCBS were retained for PCR confirmation (**Figure 3.25).** Regarding the CHROMagar™ colonies, the negative result on the oxidase test allowed to rule out the blue colonies as possible *Vibrio* spp. Nevertheless, in this experiment, to confirm the method, these colonies were also retained for PCR (**Figure 3.26**), expecting a negative result for the three targeted *Vibrio* spp. Due to its inability to grow on SNA, the mauve colonies on CHROMagar™ were only confirmed by PCR (**Figure 3.26**). Additionally to the ISO 21872-1:2017 method, since the colorless colonies on CHROMagar™ plates can correspond to *V. alginolyticus,* another pathogenic *Vibrio* species, they were retained for PCR confirmation (**Figure 3.25).** Regarding the colonies obtained on the sample analysis, all tested colonies from TCBS and CHROMagar™ plates presented a negative result for any of the three species and for the genus *Vibrio*. For the CHROMagar™ blue colonies, a small faded band was obtained for *V. vulnificus*. However, since the size obtained does not correspond to the specific *V. vulnificus* size for that target, it can be ruled out as *V. vulnificus* and explained by non-specific amplification. Considering the results obtained, none of the colonies corresponded to the three *Vibrio* spp. targeted by the ISO 21872-1:2017. Hence, the conclusion of this analysis is that, as previously stated by the reference laboratory, "Potentially enteropathogenic *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus* were not detected in 25 g of the biomass paste according to the ISO 21872-1:2017".

Additionally to the ISO 21872-1:2017 method, due to the added genus *Vibrio* primers, it is possible to infer that the contaminants that originated the colonies on TCBS and CHROMagar™ do not belong to the genus *Vibrio*. Although, it has to be taken into consideration that the primers targeting the genus *Vibrio* are not required by ISO 21872-1:2017. The method in the mentioned ISO does not detect all species of the genus *Vibrio* but merely the three ones specified: *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus.* Henceforth, the information given by the genus *Vibrio* primers is merely additional and cannot, for itself, officially conclude the presence or absence of *Vibrio* in the sample.

Nevertheless, even if the PCR done directly to the secondary enrichment broth at 37 °C, did not detect either of the three species targeted, it detected the genus *Vibrio* in the sample. Considering the great number of false positives and taking into consideration that only 5 colonies from each type and each medium proceed to further confirmation, it is understandable why results from both methods could differ. Even more, it could be a consequence of VBNC cells detected with the PCR done directly to the secondary enrichment, that are not perceived with the official method. Additionally, it is also important to consider that not all bacteria belonging to the genus *Vibrio* are pathogenic and, therefore, the detection of bacteria within the genus *Vibrio* does not mean, for itself, a threat to the human health.

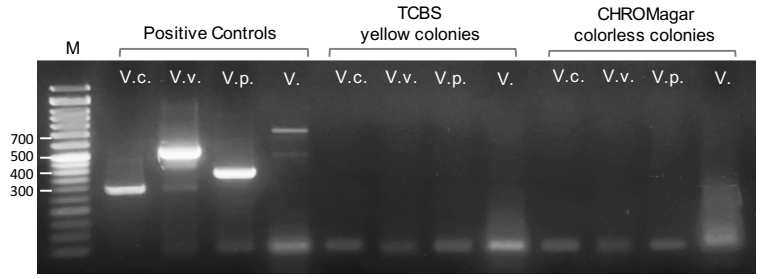


Figure 3.25 - Electrophoresis gel (2 % agarose) for the PCR products of the TCBS yellow colonies and CHROMagar™ colorless colonies. **M:** NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

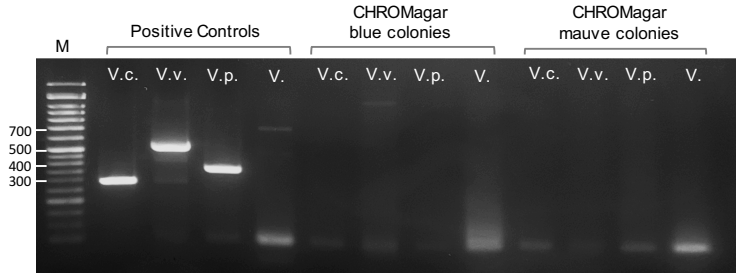


Figure 3.26 - Electrophoresis gel (2 % agarose) for the PCR products of the CHROMagar™ blue and mauve colonies. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

To sum up, we can infer that TCBS and CHROMagar™, stated as highly selective and differential media, are not as efficient as specified. Even though CHROMagar™ Vibrio is mentioned as a complementary medium that has greater efficiency in the distinction between species of *Vibrio* and other species when compared to TCBS, it allows the growth of false positive contaminants (Messelhäusser *et al.*, 2010). Even with the amendments of taking sample from the surface, both media allow the growth of microorganisms other than *Vibrio* spp., which results in a high number of false positive colonies. Thus, on TCBS it is not possible to distinguish sucrose fermenting *Vibrio* spp. from other sucrose fermenting microorganisms, possibly *Proteus* and *Aeromonas*, such as *Aeromonas hydrophila* (Donovan & Netten, 1995; FDA-BAM, 2004). The presence of these microorganisms is logical since, similarly to *Vibrio* spp., *Proteus* spp. and *Aeromonas* spp. are present in aquatic habitats, especially salt water. Therefore, they represent one possible contaminant present in microalgae cultures (Holmes *et al.*, 1996; Sabbuba *et al.*, 2003). With one confirmation step, it is possible to differentiate *Vibrio* spp. from some of the false positives. For example, *Vibrio* spp. give a positive result in the oxidase test, contrary to *Proteus* spp., which show a negative result (ISO 21872-1: 2017; Steel, 1961). Hence, the blue colonies detected on CHROMagar™ that gave a negative result for oxidase could be *Proteus.* However, other Gram-negative false positives that give a positive result for the oxidase test, such as the yellow colonies obtained on TCBS, which could be *A. hydrophila*, persist and further PCR confirmation is required in

order to confirm that those colonies are not *Vibrio* spp. (FDA-BAM, 2004). Regarding the the purple strains from CHROMagar™ Vibrio, a previous study performed with zooplankton, mussels and seawater assigned the wide range of false positives as *Aeromonas hydrophila*, *Vibrio fluvialis*, *Providenza rettgeri*, *Escherichia fergusonii*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. On TCBS agar they were identified as *Providenza rettgeri*, *Proteus mirabilis*, *Proteus vulgaris*, *Vibrio alginolyticus*, *Morganella morganii* and *Aeromonas hydrophila* (Blanco-Abad *et al.*, 2009). These only represent a niche of the possible false positive colonies.

3.3.3 ISO 21872-1:2017 method - final corrections

In the previous experiment, some setbacks were verified such as *V. cholerae* colonies smaller than expected and the absence of individual colonies on CHROMagar™ sample plates. The non-detection of the genus *Vibrio* band on the PCR positive control and the nonexistence of a Gram-positive and oxidase negative control were also verified and promptly amended in this assay.

3.3.3.1 Positive Controls

Figure 3.27; Figure 3.28 and **Figure 3.29** present the positive controls plates. The conclusions from the previous experiment regarding the positive controls morphology and the selective enrichment efficiency remain unchanged. Once again, fewer and smaller colonies of *V. cholerae* on TCBS were obtained, culminating with the decision that in the next assay, positive controls should be obtained from new frozen stocks.

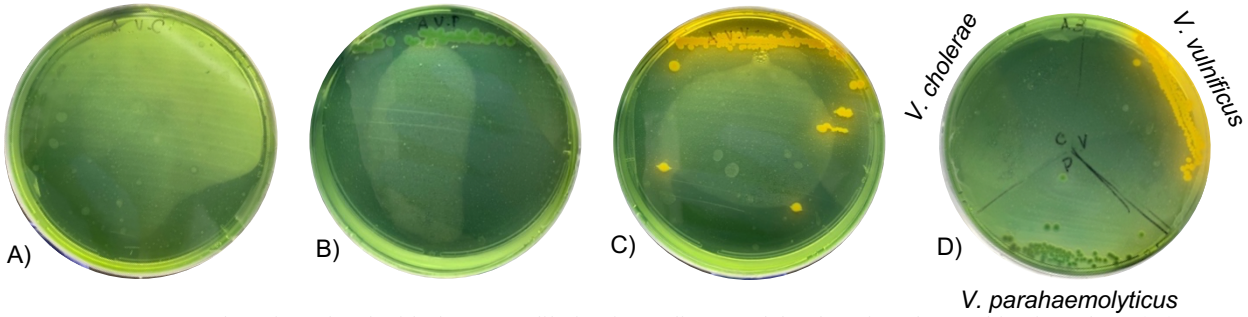


Figure 3.27 - TCBS plates inoculated with the 1:100 dilution in medium used for the microalgae cultivation of *V. cholerae* (A), *V. parahaemolyticus* (B) and *V. vulnificus* (C) and the three species on the same plate (D).

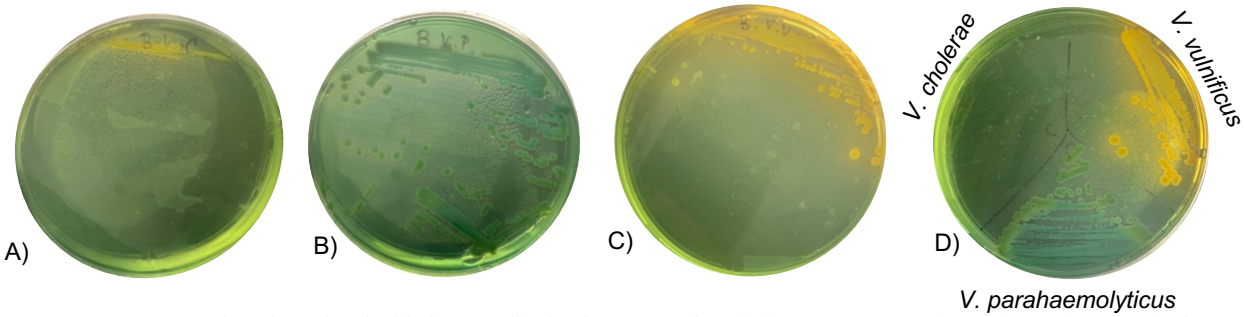


Figure 3.28 - TCBS plates inoculated with the 1:10 dilution in ASPW of *V. cholerae* (A), *V. parahaemolyticus* (B), *V. vulnificus* (C) and the three species on the same plate (D).

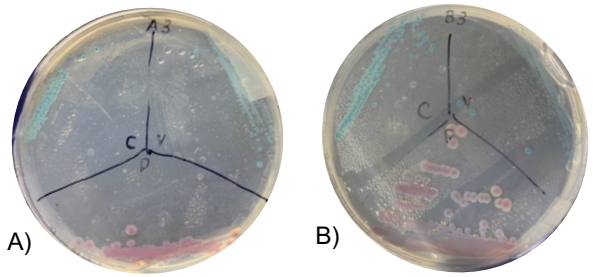


Figure 3.29 - CHROMagar™ plates inoculated with the three species 1:100 dilution in medium used for the microalgae cultivation (A) and 1:10 dilution in ASPW (B).

3.3.3.2 Detection of *Vibrio* spp. in the sample

On the **first isolation** plates, no colonies were detected on TCBS or CHROMagar™. The **second isolation** TCBS plates inoculated with the second enrichment incubated at 37 °C (**Figure 3.30A** and **Figure 3.30B**) and at 41.5 °C (**Figure 3.31A** and **Figure 3.31B**) presented one type of colonies, yellow smooth colonies with less than 1 mm in diameter. No differences were identified between the plates inoculated with the second enrichment incubated at 37 °C or 41.5 °C. These colonies were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. cholerae* or *V. vulnificus* according to the control obtained during this experiment. On CHROMagar™, the plates inoculated with the second enrichment incubated at 37 °C (**Figure 3.30C** and **Figure 3.30D**) and 41.5 °C (**Figure 3.31C** and **Figure 3.31D)** did not present any differences. For both temperatures, two types of colonies were detected, isolated colorless colonies and an agglomerate of mauve colonies on the top of the plate, with no isolated colonies. The colorless colonies were discarded as *V. cholerae, V. vulnificus* or *V. parahaemolyticus.* The colonies on the mauve agglomerate were considered presumptive *Vibrio* spp. due to their similarity with the typical color of *V. parahaemolyticus*. When comparing results from both media, the presumptive *Vibrio* spp. colonies obtained on each medium differed, which could be an indicator that these colonies do not correspond to the targeted *Vibrio* spp. Additionally, in the previous assay the same setback with no isolated mauve colonies on CHROMagar™ was verified. The streak-plate technique was implemented but the problem persisted, which confirms that the mauve colonies obtained did not grow to form detectable isolated colonies within the 24 h incubation established by the ISO 21872-1:2017, but merely after 48 h of incubation. The range difference between the size of the colonies after 24 h of incubation could, for itself, indicate that these colonies are not *Vibrio* spp. Although, similar to the previous assay, to confirm the method, they still proceeded directly to PCR confirmation.

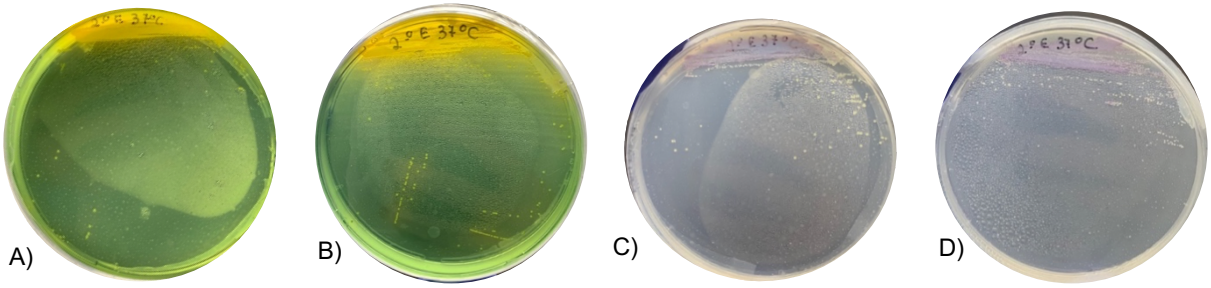


Figure 3.30 - Duplicates of TCBS plates (A) and (B) and of CHROMagar™ plates (C) and (D) inoculated with the second enrichment incubated at 37 °C.

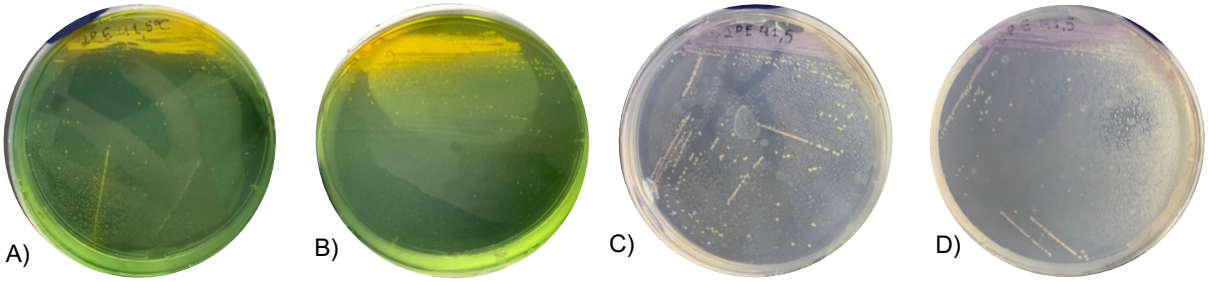


Figure 3.31 - Duplicates of TCBS plates (A) and (B) and of CHROMagar^{TM} plates (C) and (D) inoculated with the second enrichment incubated at 41.5 °C.

Regarding the **confirmation step (tests for presumptive identification),** it was implemented a Gram-positive and oxidase negative control to confirm the results obtained, *Listeria ivanovii* - CECT 913^{T}. All the isolates on TCBS presented a pink to red color for Gram staining, concluding they are all Gram-negative microorganisms like *Vibrio* spp. **Figure 3.32** presents the Gram-negative (*V. cholera*), the Gram-positive (*Listeria ivanovii)* and one isolate from TCBS.

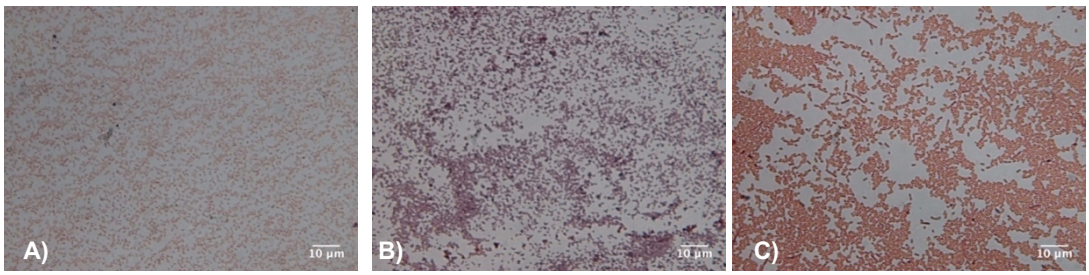


Figure 3.32 - Gram staining results for *V. cholerae* Gram-negative control (A), *Listeria ivanovii* Gram-positive control (B) and one isolate from the TCBS yellow colonies.

Regarding the oxidase test, like *Vibrio* spp. positive controls, all the isolates on TCBS presented a positive result detecting cytochrome oxidase (**Figure 3.33**). Hence, the results obtained with these two tests did not allow the discrimination of the false positives obtained on TCBS which led to the requirement of the next confirmation step, PCR.

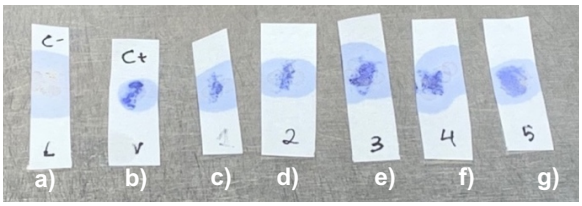


Figure 3.33 - Oxidase test results for *Listeria ivanovii* oxidase negative control (a), *V. cholerae* oxidase positive control (b), and five isolates from TCBS yellow colonies (c-g).

Concerning the **confirmation step through PCR, Figure 3.34** presents the PCR done to screen for the presence of *Vibrio* spp. in the second enrichment broth at 37 °C, following incubation. Two faded bands were detected for the target *V. vulnificus.* Although, these bands presented different sizes than the product expected for *V. vulnificus*, approximately 450 bp and 1000 bp, instead of 519 bp. Consequently, it was possible to infer that there was no *V. parahaemolyticus, V. cholerae* or *V. vulnificus* in the second enrichment broth. Additionally, a faded band was detected for the genus *Vibrio*, evidencing the presence of contaminants belonging to the genus *Vibrio* other than the three species targeted individually. Regarding this primer set, to rectify the nonappearance of the genus *Vibrio* band for the positive control, a master mix was prepared for all the reactions together and the number of cycles was increased from 25 to 30 cycles. As seen in **Figure 3.34** and **Figure 3.35,** with the increasement of cycles, an intense band with the expected size (689 bp) was obtained. Although, another nonspecific band, with

approximately 450 bp, was observed. This secondary band could be explained by exogenous contamination or unspecific binding due to excess of DNA or cycles, also noticeable for the *V. parahaemolyticus* positive control (Altshuler, 2006).

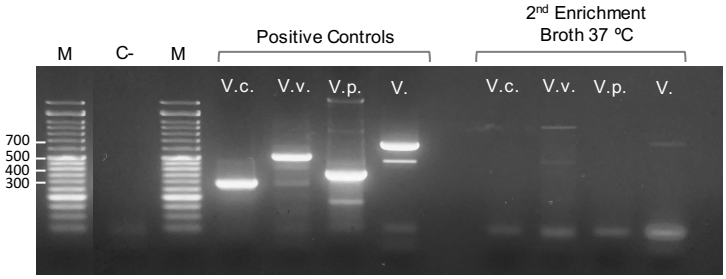


Figure 3.34 - Electrophoresis gel (2% agarose) for the PCR products of the second enrichment broth at 37 °C. **M**: NZY Ladder VI (NZYtech); **(V.c.)**: *V. cholerae*; **(V.v.)**: *V. vulnificus*; **(V.p.)**: *V. parahaemolyticus*; **(V.)**: genus *Vibrio*; **(C-)**: negative control. The primers target is written in white and the sample in test is indicated in black.

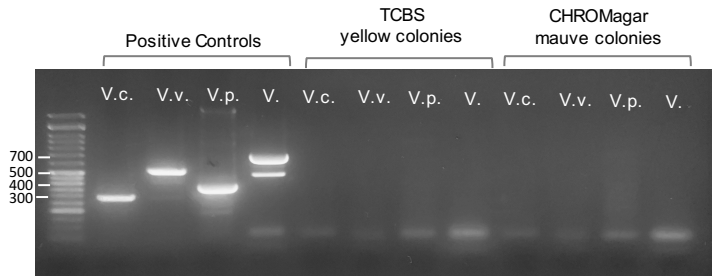


Figure 3.35 - Electrophoresis gel (2% agarose) for the PCR products of the TCBS yellow colonies and CHROMagar™ mauve colonies. **M**: NZY Ladder VI (NZYtech); **(V.c.)**: *V. cholerae*; **(V.v.)**: *V. vulnificus*; **(V.p.)**: *V. parahaemolyticus*; **(V.)**: genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

Regarding the colonies obtained in the sample analysis, either the yellow colonies detected on TCBS and the mauve colonies obtained on CHROMagar™ plates presented a negative result for any of the three *Vibrio* spp. targeted by the ISO 21872-1:2017 and for the genus *Vibrio*. Considering the results obtained, the conclusion of this analysis is that "Potentially enteropathogenic *V. cholerae, V. parahaemolyticus* and *V. vulnificus* were not detected in 25 g of the paste according to the ISO 21872-1:2017", in agreement with the result previously obtained by the reference laboratory and the screening of the enrichment broth. Additionally to the ISO method, due to the added genus *Vibrio* primers, we can also infer that the contaminants that originated the targeted colonies on TCBS and CHROMagar™ do not belong to the genus *Vibrio*. Nevertheless, like in the previous experiment, the PCR done directly to the secondary enrichment broth at 37 ºC, following incubation, detected the genus *Vibrio* in the sample. Even though, as previously stated, not all bacteria belonging to the genus *Vibrio* are pathogenic and, therefore, the detection of bacteria within the genus *Vibrio* does not mean, for itself, a threat to the human health.

3.4 Spiked Samples

The last step consisted of using spiked samples with *Vibrio* spp. to test if the method could detect it or if the high number of false positives, previously obtained, would mask the true positives leading to false conclusions.

3.4.1 CFU inoculated into the spiked samples

Table 3.2 presents the values obtained for the CFU counts on dilutions 10^{-6} and 10^{-7} from the overnight growth cultures. According to the correlation previously obtained **(Figure 3.2)** these dilutions correspond to approximately 100 and 10 CFU, respectively. Even though CFU should only be counted on plates presenting between 30 and 300 colonies (Maier *et al*., 2000), the values corresponding to the 10^{-7} dilution were included in the table for additional information purpose. Though the values present some differences between them, the order of magnitude is reasonably within the expected values of 100 and 10 CFU. Considering the values obtained, 100 µL of the 10^{-6} dilution inoculated into the Schott represented an inoculum of around 62 - 233 CFU. Or 5 - 25 CFU when the 10^{-7} dilution was considered. The fact that *V. cholerae* presented higher CFU mL^{-1} could be a result that the LB media formulation used along these experiments was retrieved from a protocol optimized for *V. cholerae* (Martinez *et al.*, 2010).

Table 3.2 - CFU counts per 100 µL for the dilutions 10^{-6} and 10^{-7} of the original sample for each *Vibrio* sp.

	Dilution	
	10^{-6}	**10^{-7}**
V. cholerae	224; 233; 227	18; 16; 25
V. parahaemolyticus	97;101;77	9; 5; 6
V. vulnificus	71; 62; 75	23;18;10

3.4.2 Positive Controls

In **Figure 3.36** the positive controls plates are presented. The conclusions from the previous experiment regarding the positive controls morphology on both media and the selective enrichment efficiency remained. In this assay, the positive controls inoculated were obtained from new frozen stocks. Although, the discrepancy within the *V. cholerae* dimensions was still verified. Considering that it grew to form colonies identical to *V. vulnificus* on CHROMagar™, a possible explanation is that this species, or at least this strain, does not grow on TCBS as well as *V. parahaemolyticus* and *V. vulnificus,* presenting smaller dimensions after 24 h of incubation. Previous studies have stated that the sodium citrate used on TCBS was inhibitory to *V. vulnificus* (Donovan & Netten, 1995). Speculating, the same problem could occur with some *V. cholerae* strains on TCBS. Considering that blue colonies with 1 mm were obtained on CHROMagar™, this setback does not affect the overall results considering that *V. cholerae* is clearly detected on CHROMagar™, supporting the need for the two selective media. It also indicates that CHROMagar™ is less inhibitory to *V. cholerae*. It is also important to consider that although the TCBS media used along the experiments as approved by ISO, two different suppliers were used. In the preliminary tests, where *V. cholerae* formed typical colonies, the supplier was HIMEDIA while from then on it was Liofilchem. Even though the two media present the exact composition, except for 5 g of Oxgall at HIMEDIA and 8 g at Liofilchem, even a small difference in composition of the media could be the cause for this difference in *V. cholerae* colonies.

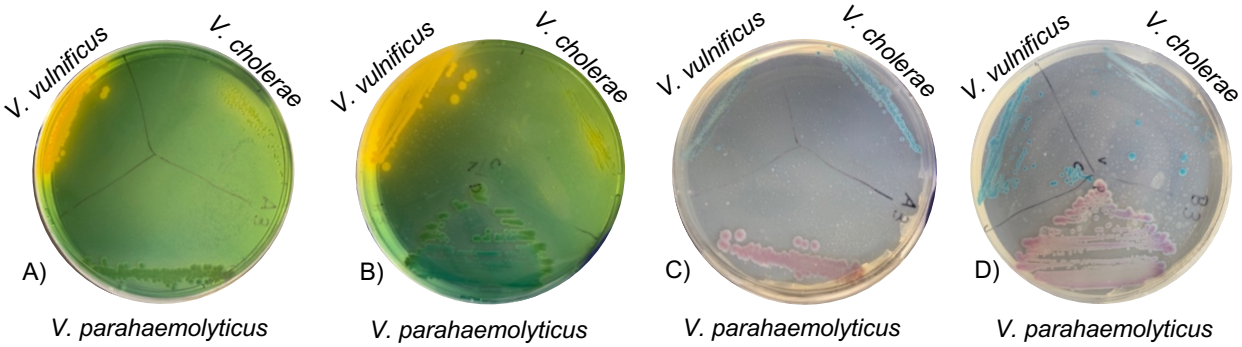


Figure 3.36 - TCBS (A) and (B) and CHROMagar™ (C) and (D) plates inoculated with the three species 1:100 dilution in medium used for the microalgae cultivation (A) and (C) and 1:10 dilution in ASPW (B) and (D).

3.4.3 Detection of *Vibrio* spp. in the sample

The results obtained after the first enrichment on TCBS or CHROMagar™ showed no colonies in both media for the negative control (sample N) and for the plates inoculated with ≈ 10 CFU (sample ≈ 10 CFU). As shown in **Figure 3.37**, regarding sample ≈ 100 CFU, smooth, mauve colonies with 2 mm in diameter were detected on CHROMagar™, typical of *V. parahaemolyticus* with the predictable dimensions and not in the form of an agglomerate, as obtained in the previous experiments. Hence, it is possible to infer that for concentrations bellow 10 CFU per 25 g of sample, a single selective enrichment is not efficient enough to detect *Vibrio* spp. Regarding concentrations around 100 CFU, CHROMagar™ was able to detect *V. parahaemolyticus* colonies, even if typical colonies of *V. cholerae* and *V. vulnificus* were not present at this stage. Even more, considering that a higher number of *V. cholerae* CFU was inoculated, it is possible that the selective enrichment or the CHROMagar™ favor *V. parahaemolyticus'* growth in comparison to *V. cholerae* or *V. vulnificus.* Additionally, the absence of colonies on TCBS for the three samples suggests that CHROMagar™ is more effective than TCBS for the detection of *Vibrio* spp. or, at least, *V. parahaemolyticus,* as occurred in this experiment and *V. cholerae,* as proven in the positive control.

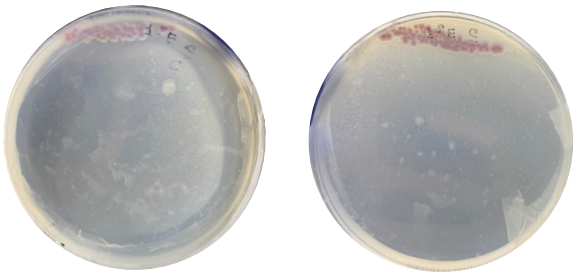


Figure 3.37 - Duplicates of CHROMagar™ plates inoculated with the first enrichment of sample ≈ 100 CFU.

The plates inoculated with the second enrichment are presented in **Figure 3.38**, **Figure 3.39**, **Figure 3.40**, **Figure 3.41**, **Figure 3.42 and Figure 3.43.**

In the **second isolation** plates of sample (N), as observed in **Figure 3.38**, on the TCBS plates inoculated with the second enrichment incubated at 37 °C and 41.5 °C one type of colonies was detected, yellow smooth colonies with less than 1 mm in diameter. Besides the higher number of yellow colonies at 41.5 °C, no differences were identified between the plates inoculated with the second enrichment incubated at both temperatures. These colonies were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. cholerae* or *V. vulnificus*, even if smaller than expected. On CHROMagar™ **(Figure 3.41)** the plates inoculated with the second enrichment incubated at 37 °C and 41.5 °C presented slight differences. For both temperatures, three types of colonies were detected,

isolated colorless colonies with less than 1 mm in diameter and an agglomerate of mauve and blue colonies on the top of the plate. At 41.5 °C a higher number of colorless colonies was detected. Even if smaller, the mauve colonies were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. parahaemolyticus* while the blue colonies with typical colonies of *V. vulnificus* and *V. cholerae.*

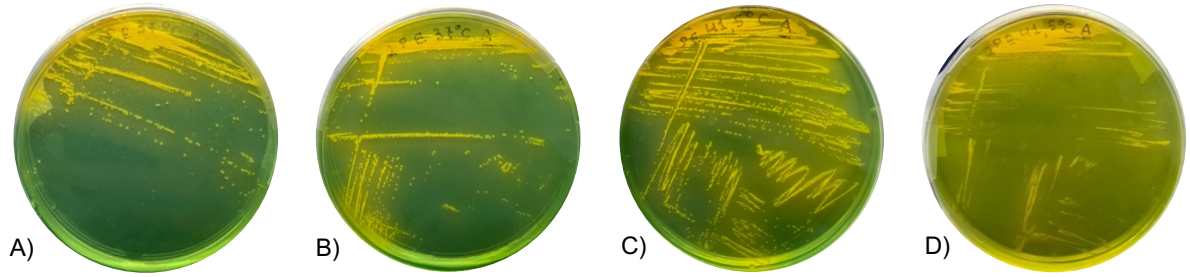


Figure 3.38 - Duplicates of TCBS plates inoculated with the second enrichment of the negative control (N) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

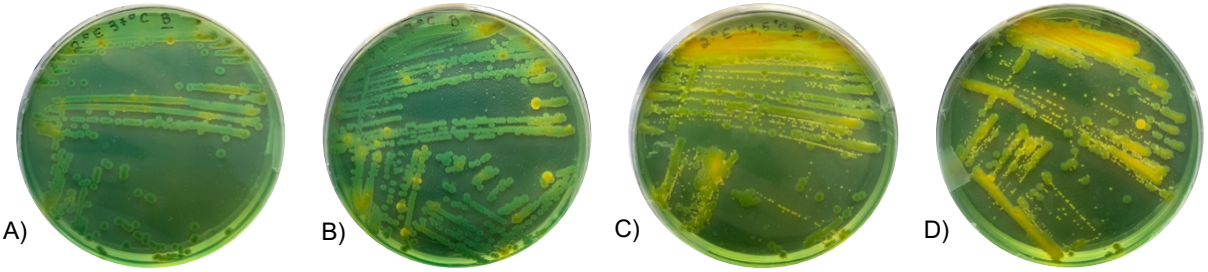


Figure 3.39 - Duplicates of TCBS plates inoculated with the second enrichment of the spiked sample (≈ 10 CFU) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

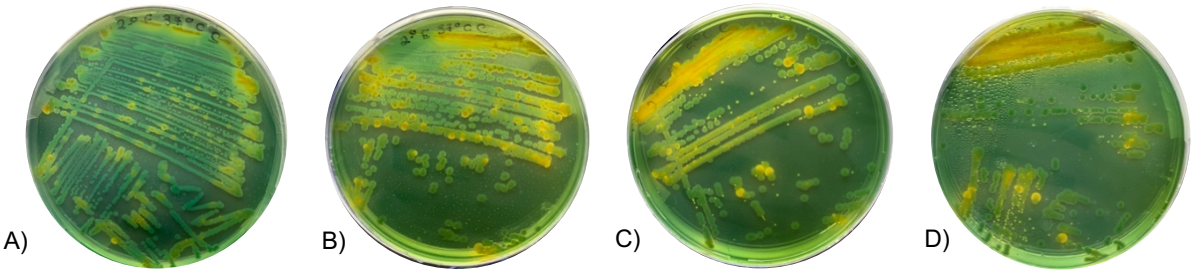


Figure 3.40 - Duplicates of TCBS plates inoculated with the second enrichment of the spiked sample (≈ 100 CFU) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

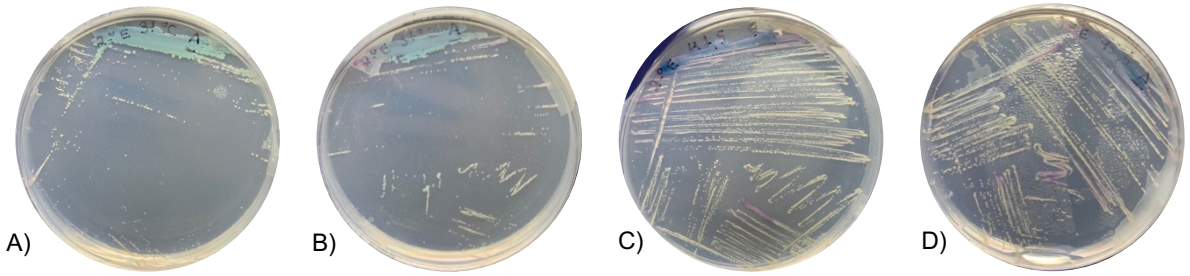


Figure 3.41 - Duplicates of CHROMagar™ plates inoculated with the second enrichment of the negative control (N) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

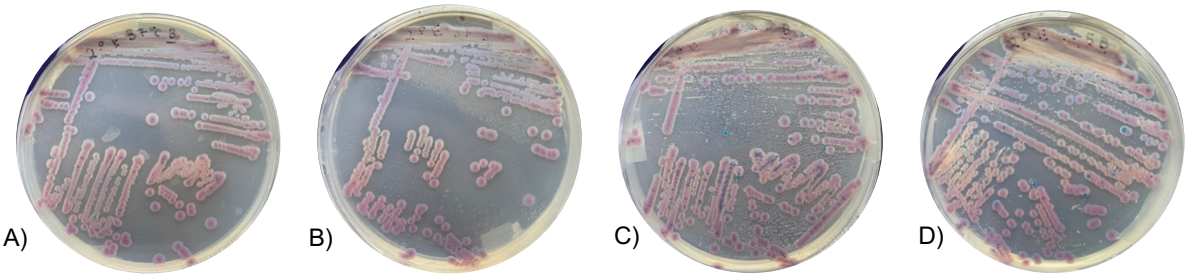


Figure 3.42 - Duplicates of CHROMagar™ plates inoculated with the second enrichment of the spiked sample (≈ 10 CFU) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

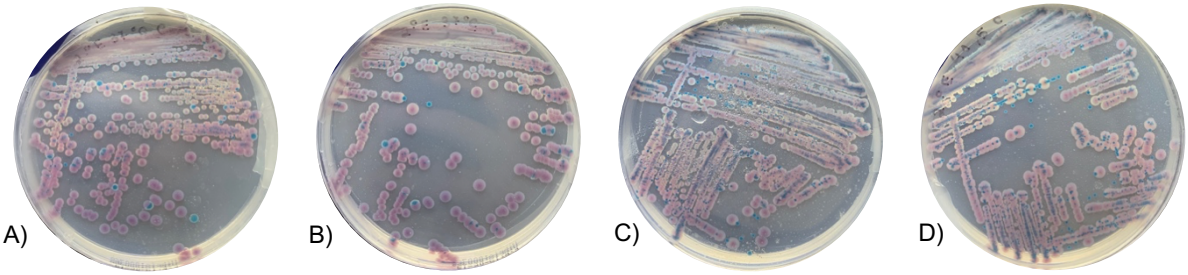


Figure 3.43 - Duplicates of CHROMagar™ plates inoculated with the second enrichment of the spiked sample (≈ 100 CFU) incubated at 37 °C (A) and (B) and 41.5 °C (C) and (D).

In the spiked sample ≈ 10 CFU, as observed in **Figure 3.39,** on the TCBS plates inoculated with the second enrichment incubated at 37 °C and 41.5 °C, two types of colonies were detected, yellow and green smooth colonies with 2 - 3 mm in diameter. Besides the higher number of yellow colonies at 41.5 °C, no differences were identified between the plates. The yellow colonies were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. cholerae* or *V. vulnificus*, including the expected size. The green colonies were considered presumptive *Vibrio* spp. due to their similarity with typical colonies of *V. parahaemolyticus*, including the predictable size. On CHROMagar™ plates (**Figure 3.42)** inoculated with the second enrichment incubated at 37 °C and at 41.5 °C three types of colonies were detected, few colorless colonies with less than 1 mm in diameter, a lot of mauve smooth colonies with 2 - 3 mm in diameter, and few smooth blue colonies with 1 - 2 mm in diameter, that were present in a higher concentration at 41.5 °C which could be explained by the fact that 41.5 °C is the most suitable temperature for *V. cholerae* detection. The mauve colonies, typical colonies of *V. parahaemolyticus* and the blue colonies, typical colonies of *V. vulnificus* and *V. cholerae* proceeded to PCR confirmation.

Regarding the spiked sample ≈ 100 CFU, as observed in **Figure 3.40,** on the TCBS plates the same conclusions taken for sample ≈ 10 CFU remained. While on CHROMagar™ (**Figure 3.43)** the same conclusions for sample ≈ 100 CFU also persisted, as expected, a higher number of smooth blue colonies with 1 - 2 mm in diameter, was detected in comparison with sample ≈ 10 CFU.

Overall, the three targeted species were identified in the spiked samples. Although, it is important to consider that *V. vulnificus* and *V. cholerae* typical colonies cannot be distinguished from each other. The colonies obtained in the spiked samples presented the predictable dimensions according to ISO, while the colonies considered as typical in the sample N and in the previous assays were smaller than expected. With these results it was possible to conclude that, even though some strains could be inhibited in one of the media, resulting in smaller colonies, the complementary between the two media would attenuate this setback. This would allow to exclude the colonies with less than 1 mm as the three targeted *Vibrio* spp. To confirm this theory, for sample N and spiked samples ≈ 10 CFU and ≈ 100 CFU, from each

medium, 5 typical colonies of each *Vibrio* sp. proceeded to further confirmation to infer if the three species could be detected.

After the previous analysis performed, it was possible to infer that Gram staining and oxidase tests do not allow the discrimination of all false positives obtained on TCBS and CHROMagar™, requiring further confirmation through PCR. Since Gram staining and oxidase tests are time-consuming, requiring previous isolation on SNA, adding 24 h to the total analysis time, it was decided to skip Gram staining and oxidase test and advance directly to PCR. Considering that all the colonies that proceed to Gram staining and oxidase test are confirmed with PCR, this method does not diverge from the ISO 21872-1:2017. Furthermore, when using the positive controls colonies obtained directly from TCBS and CHROMagar™ for PCR, it is possible to infer the efficiency of the method and that no inhibitions occur.

Regarding the **confirmation step through PCR, Figure 3.44, Figure 3.45** and **Figure 3.46** present the results of the PCRs done to screen for the presence of *Vibrio* spp. in the second enrichment broth of sample N, ≈ 10 CFU and ≈ 100 CFU, respectively. Firstly, the genus *Vibrio* positive control did not amplify in any of the samples. In the PCR screening of the sample N (**Figure 3.44)** no bands were present for *V. parahaemolyticus.* Contrarily, nonspecific amplification occurred for the *V. cholerae* and *V. vulnificus* primer sets both at 37 °C and 41.5 °C. For *V. cholerae* only one band was present with approximately 1200 bp, clearly different from the 295 - 310 bp expected. For *V. vulnificus* a variety of bands was obtained. Two of these bands were intense and clearly differed from the 519 bp product expected for this species, another two faded bands were presented with sizes around 519 bp but not precisely. Hence, the screening of the sample N allowed to infer the absence of all three targeted species. Even if genus *Vibrio* positive control did not amplify, at 37 °C a band with less than 700 bp was obtained, near the 689 bp expected for the genus *Vibrio*, detecting other microorganisms belonging to the genus *Vibrio*. The results of this screening are in accordance with the expected result of absence of the targeted *Vibrio* spp. in the sample. Regarding the screening of the sample ≈ 10 CFU (**Figure 3.45)** and ≈ 100 CFU **(Figure 3.46),** the results were similar. The genus *Vibrio*, *V. parahaemolyticus* and *V. vulnificus* were detected in both samples at 37 °C and 41.5 °C. Contrarily, *V. cholerae* was only detected in the sample ≈ 100 CFU at 41.5 °C. Even though it was still expected to detect *V. cholerae* in the sample ≈ 10 CFU, the fact that it was only detected at 41.5 °C in the sample ≈ 100 CFU is in accordance with the fact that 41.5 °C is the chosen temperature for *V. cholerae* detection according to the ISO 21872-1:2017.

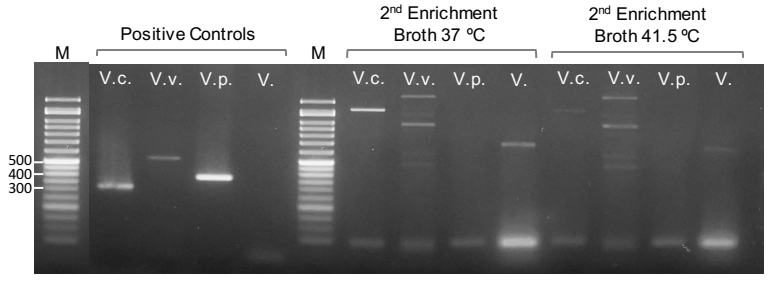


Figure 3.44 - Electrophoresis gel (2% agarose) for the PCR products of the second enrichment broth of sample N. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

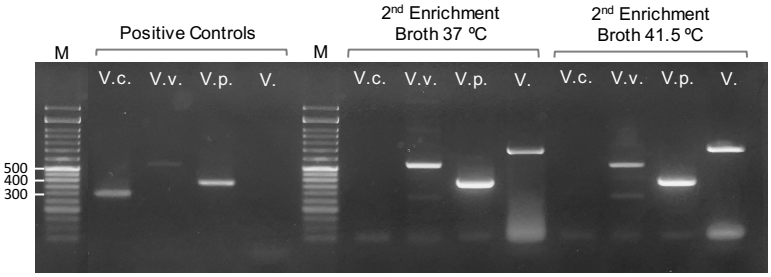


Figure 3.45 - Electrophoresis gel (2% agarose) for the PCR products of the second enrichment broth of sample ≈ 10 CFU. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

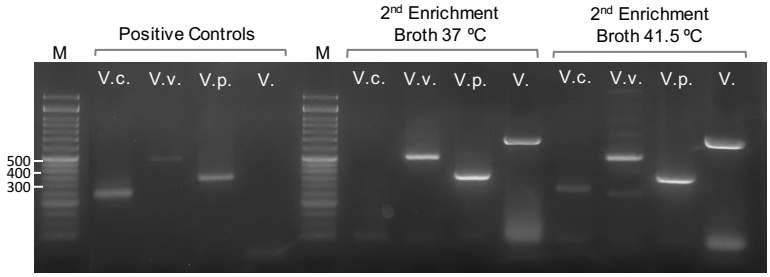


Figure 3.46 - Electrophoresis gel (2% agarose) for the PCR products of the second enrichment broth of sample ≈ 100 CFU. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

Figure 3.47 presents the electrophoresis gel with the PCR products from the colonies obtained in the spiked sample ≈ 10 CFU. There were a significant number of unspecific bands, especially for *V. vulnificus* and the same problem was observed for all the samples (results not presented). As stated by Garibyan and Avashia (2013), the specificity of the generated PCR product may be altered by nonspecific binding of the primers to other similar sequences on the template DNA. Using a higher concentration of DNA combined with normal, or higher number of cycles can result in the quicker accumulation of nonspecific products (Altshuler, 2006). Indeed, too much DNA was present in the reaction as a consequence of excessive inoculum during DNA extraction. This can be explained by the fact that, unlike in the previous experiments, an inoculating loop was used in place of micropipette tips, which lead to an increased amount of inoculum into the extraction tube.

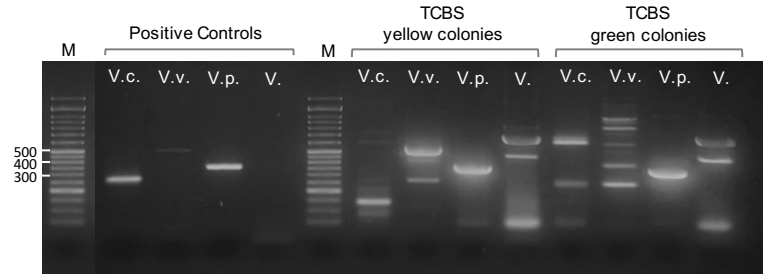


Figure 3.47 - Electrophoresis gel (2% agarose) for the PCR products of the TCBS yellow colonies and TCBS green colonies obtained in sample ≈ 10 CFU. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*; (**V.**): genus *Vibrio*. The primers target is written in white and the sample in test is indicated in black.

To correct the excess of DNA upon the DNA extraction, after testing dilutions ranging from 1:10 to 1:1000 (results not presented), the 1:1000 dilution was performed for every sample and the PCRs were repeated. Considering the previous setbacks with the genus *Vibrio* primer set and that the information provided by this primer is not required by the ISO 21872-1:2017 along with the fact that a positive result does not mean, for itself, a threat to human health, this primer was withdrawn from the method and only the three targeted species were tested.

Figure 3.48, **Figure 3.49**, **Figure 3.50**, and **Figure 3.51** present the electrophoresis gels with the PCR products of the colonies obtained in the sample N, ≈ 10 CFU and ≈ 100 CFU. Regarding the colonies obtained in the sample N analyses, like occurred in the screening, unspecific binding was still obtained for *V. vulnificus* with two faded bands with sizes around 519 bp but not precisely (**Figure 3.48**). Conclusively, all tested colonies presented a negative result for any of the three *Vibrio* spp. targeted by the ISO 21872-1:2017, in agreement with the result previously obtained by the reference laboratory. These results are also in accordance with the PCR screening for the second enrichment broth of the sample N (**Figure 3.44).** For the PCR of the colonies obtained in the sample ≈ 10 CFU (**Figure 3.49** and **Figure 3.50**) and ≈ 100 CFU (**Figure 3.50** and **Figure 3.51**), similar results were obtained for both samples with only slight differences for *V. cholerae* detection. As expected for both samples, CHROMagar™ mauve colonies, presumptive *V. parahaemolyticus*, presented a positive result for this species. Additionally, an unspecific band was observed for *V. vulnificus* with a size differing from the target one. Regarding CHROMagar™ blue colonies, presumptive *V. cholerae* and *V. vulnificus,* both species were detected for the two samples which is in accordance with the expected results. Additionally, *V. parahaemolyticus* was also detected in sample ≈ 10 CFU due to the inability to obtain blue colonies apart from the mauve colonies.

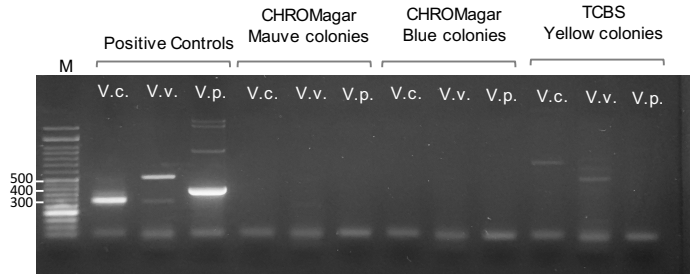


Figure 3.48 - Electrophoresis gel (2% agarose) for the PCR products of the CHROMagar™ mauve and blue colonies and the TCBS yellow colonies obtained in sample N. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*. The primers target is written in white and the sample in test is indicated in black.

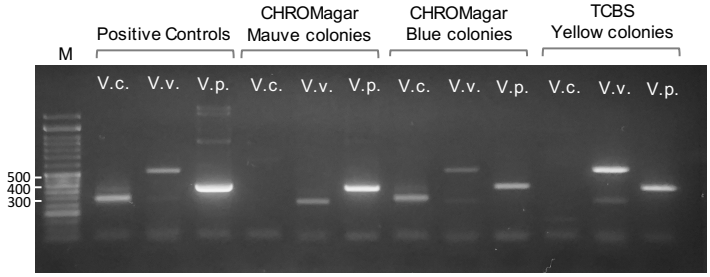


Figure 3.49 - Electrophoresis gel (2% agarose) for the PCR products of the CHROMagar™ mauve and blue colonies and the TCBS yellow colonies obtained in sample ≈ 10 CFU. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*. The primers target is written in white and the sample in test is indicated in black.

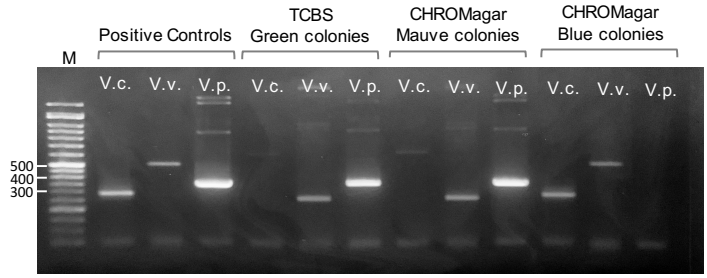


Figure 3.50 - Electrophoresis gel (2% agarose) for the PCR products of the TCBS green colonies obtained in sample ≈ 10 CFU and CHROMagar™ mauve and blue colonies obtained in sample ≈ 100 CFU. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*. The primers target is written in white and the sample in test is indicated in black.

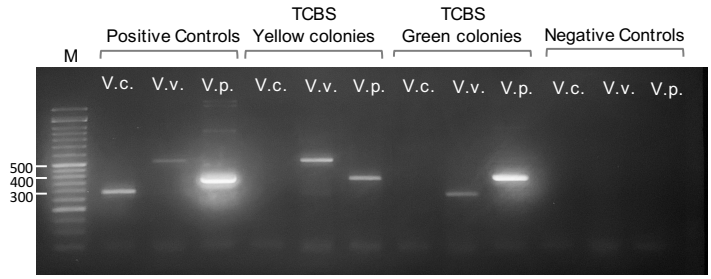


Figure 3.51 - Electrophoresis gel (2% agarose) for the PCR products of the TCBS yellow and green colonies obtained in sample ≈ 100 CFU and negative controls for each primer set. **M**: NZY Ladder VI (NZYtech); (**V.c.**): *V. cholerae*; (**V.v.**): *V. vulnificus*; (**V.p.**): *V. parahaemolyticus*. The primers target is written in white and the sample in test is indicated in black.

Considering the colonies on TCBS, the green ones correspond to presumptive *V. parahaemolyticus* (**Figure 3.50** and **Figure 3.51**). Similar to the CHROMagar™ mauve colonies, a positive result was obtained for this species and an unspecific band was observed for *V. vulnificus* with a size differing from the target size. The yellow colonies (**Figure 3.51**) represent presumptive *V. cholerae* and *V. vulnificus.* For both spiked samples, *V. parahaemolyticus* was detected due to the inability to obtain yellow colonies away from the green colonies and amplification occurred as expected for *V. vulnificus* but not for *V. cholerae*. Two theories could explain this fact. First is that both *V. vulnificus* and *V. cholerae* were present on the plate, with 2 - 3 mm colonies, but the 5 colonies picked were only *V. vulnificus.* The second option, and the most likely, is that *V. cholerae* did not originate typical colonies on TCBS with 2 - 3 mm, similarly to the positive control, but only colonies with 1 mm or less. Like in the positive control, *V. cholerae* originated the blue colonies with around 1 - 2 mm on CHROMagar™ which confirmed the higher efficacy of this medium to detect this species or at least this strain of *V. cholerae.* The confirmation that even though some strains could be inhibited in one of the media, resulting in smaller colonies, but grow to form typical colonies in the second medium, confirms the importance of the complementarity between the two media as previously stated by other authors (Rosec *et al*., 2012).

The results obtained with the PCR screening either from the obtained colonies either directly from the second enrichment broth (**Figure 3.45**; **Figure 3.46)** were mainly in accordance, except for *V. cholerae.* This species was detected on the mauve CHROMagar™ colonies for sample ≈ 10 CFU and ≈ 100 CFU but only detected in the screening at 41.5 °C for sample ≈ 100 CFU with a faded band. Firstly this can be a result that although *V. cholerae* had the higher CFU inoculated into the spiked samples, the selective enrichment favored the other two species growth comparing to *V. cholerae*. This occurrence is possibly due to the fact that even though *V. cholerae* is able to grow at concentrations up to 6 % (m V^{-1}) NaCl, it does not require salt for growth (Bhunia, 2018). Therefore, the amount of salt in the ASPW, 2 % (m V^{-1}) NaCl, could favor the optimum growth of the other two species that actually require salt. Although for *V. cholerae* and *V. parahaemolyticus* [1 - 9 % (m V^{-1}) NaCl] the values presented in the literature are only for survival and not for optimum growth, it is stated that *V. vulnificus* has an optimum salt requirement of 1 to 2 % (m V^{-1}) NaCl which is in accordance with the fact that *V. vulnificus* thrived in ASPW (Bhunia, 2018; Yeung & Boor, 2004).

Concluding, for both spiked samples the tested colonies presented a positive result for the three *Vibrio* spp. targeted by the ISO 21872-1:2017. Hence, the conclusion of this analysis is that "Potentially enteropathogenic *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus* were detected in 25 g of the paste according to the ISO 21872-1:2017". This conclusion is in agreement with the expected results considering that these samples were spiked with the three *Vibrio* species. This allows to infer that even though a high number of false positive colonies is present, the difference in dimensions between the false positives and the typical colonies along with the complementarity of both media allows to detect the three targeted *Vibrio* spp. and to identify false positives considering their difference in size.

3.5 Financial analysis

A financial analysis was performed to understand the offsets of implementing the method at A4F rather than asking a reference laboratory to perform the analysis. Firstly it was important to consider that the reference laboratory never had to perform the confirmation step, merely doing the first and second enrichment steps, isolation and identification to conclude the absence of *Vibrio* spp. in the sample. This fact is in accordance with the previous conclusions, in the spiked samples, that the colonies smaller than 1 mm can be ruled out as *Vibrio* spp. without additional confirmation. Hence, the financial analysis was performed for the ISO 21872-1:2017 method including all the steps but no step for confirmation. The financial analysis was calculated to an industrial scale with a significant number of analysis performed in a row. Therefore, preparation of material and media would be done one time and used in several analysis, saving time. Work hours presented, material preparation and cleaning of the biosafety chamber were already included in the values presented and the costs associated with this section were calculated considering the average salary of a biology/biotechnology technician.

3.5.1 Preparation of the positive controls

Figure 3.52 presents the possible cutbacks, inoculating the three species on the same plate and only using TCBS to infer the efficiency of the selective enrichment.

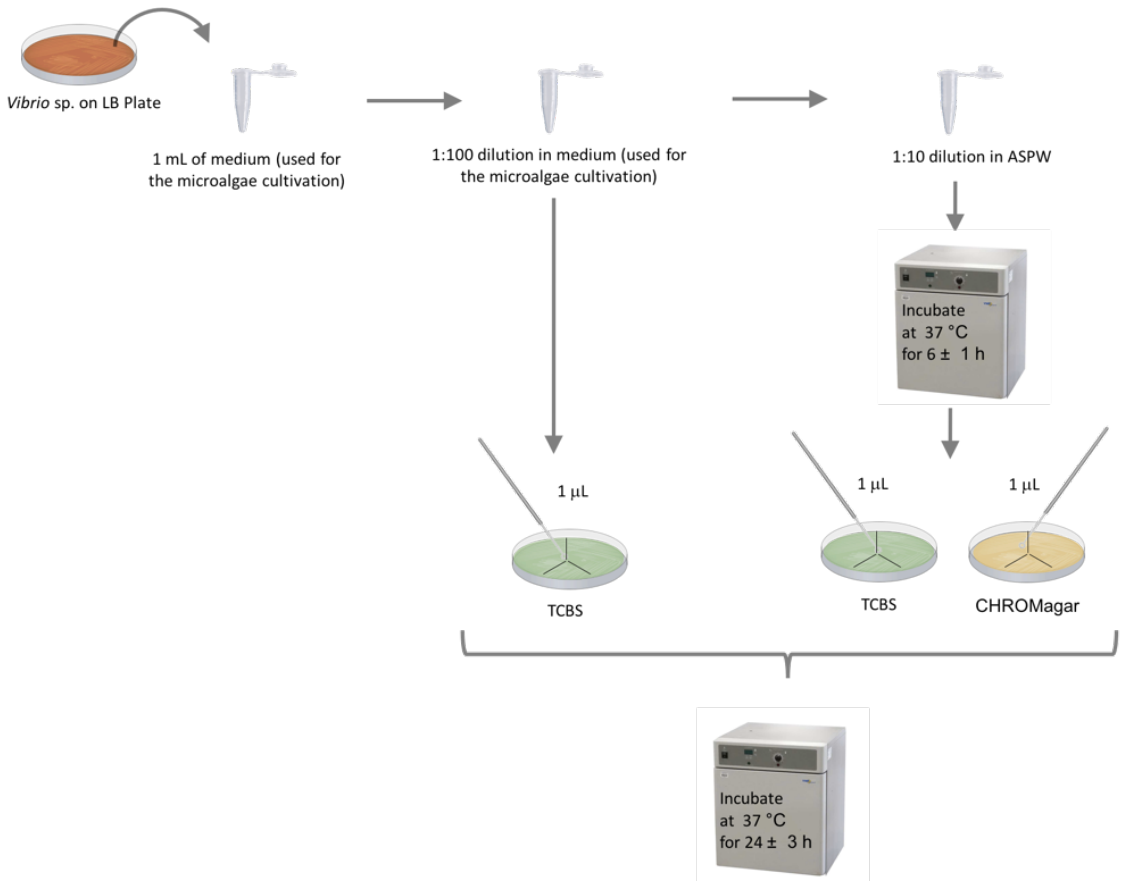


Figure 3.52 - Preparation of the positive controls (A4F's method) after possible cutbacks.

Table 6.5 in appendix presents the price of each material required for the analysis. **Table 3.3** presents the number of each material required for this step and total prices. Negative controls for each medium were already included in the total number of plates required.

Table 3.3 - Financial analysis for the preparation of the positive controls (A4F's method) after possible cutbacks. The prices presented do not include Value Added Tax (VAT) to allow a direct comparison with the reference laboratory price provided for each analysis.

Product	Amount	Total price (€)
1.5 mL micro-centrifuge tubes	9	0.063
1 µL inoculating loops	12	0.252
10 µL filter tips	3	0.131
100 µL filter tips	3	0.131
1000 µL filter tips	11	0.650
ASPW (mL)	6	0.009
CHROMagar™ Vibrio (plates)	2	2.976
T.C.B.S. Agar (plates)	3	0.546
Work hours (h)	2.5	15.208
Total		19.996

3.5.2 Standard ISO 21872-1:2017 method - first and second enrichment

Figure 3.53 presents the official ISO 21872-1:2017 method for the sample analysis. Considering that usually no or few colonies were detected in the first enrichment, one plate divided into two can be used.

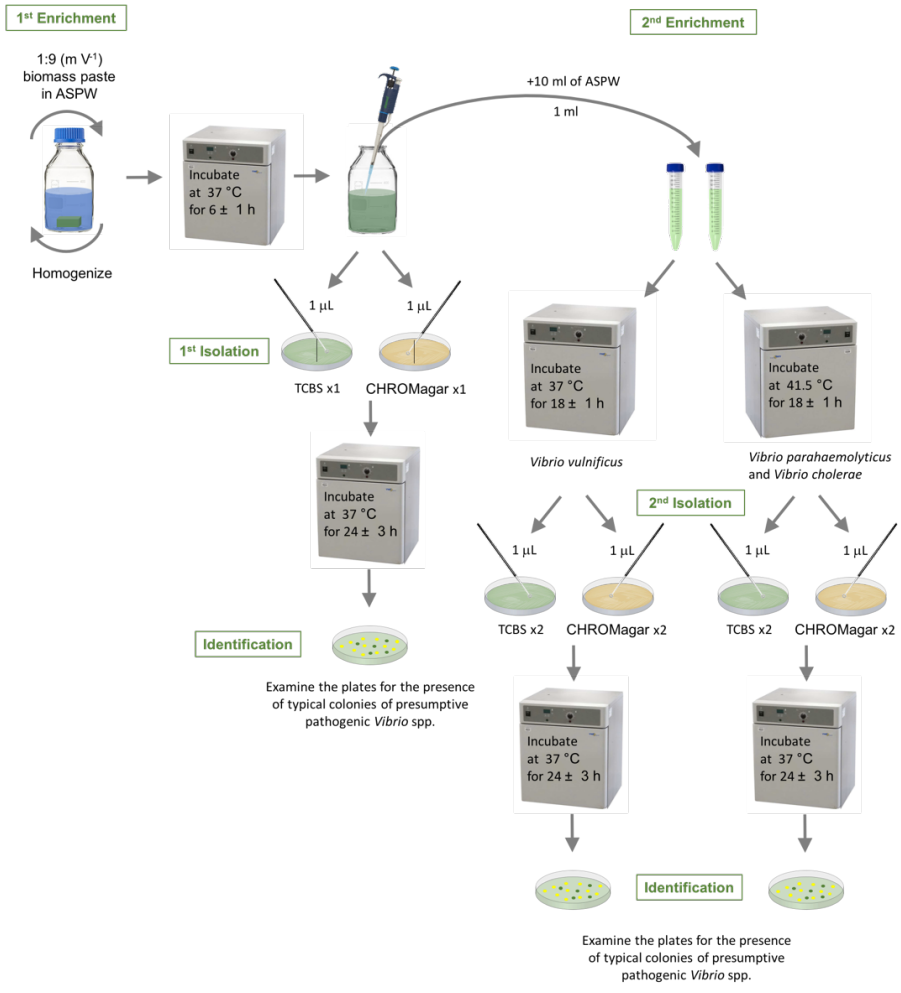


Figure 3.53 - Official ISO 21872-1:2017 method for the samples analysis after possible cutbacks.

In **Table 3.4** the financial analysis for the first and second enrichment step is presented.

Table 3.4 - Financial analysis for the official ISO 21872-1:2017 method for the analysis of the sample after possible cutbacks. The prices presented do not include VAT to allow a direct comparison with the reference laboratory price provided for each analysis.

Product	Amount	Total price (€)
Cell scraper	2	1.360
ASPW (mL)	245	0.372
1000 µL filter tips	2	0.118
10 mL graduated pipettes	1	0.140
1 µL inoculating loops	10	0.210
15 mL falcon tubes	2	0.156
CHROMagar™ Vibrio plates	5	7.440
T.C.B.S. Agar plates	5	0.910
Work hours (h)	3.5	21.291
Total		31.997

Considering that the analysis would be done in an industrial scale production, analysis could be done together in order to dilute the positive control cost between all the samples. If three samples were tested together, the cost for each sample would be 39 euros, more than three times higher than the price provided by the reference laboratory.

Chapter 4 - Conclusion and Future Perspectives

With the increasing microalgae production at A4F, the main goal of this work was to implement the ISO 21872-1:2017 method to detect *Vibrio* spp. in microalgae biomass, satisfying the customers' request for a standard method. More specifically, the final goal was to infer if it offsets to implement the method at A4F's laboratory or if it is preferable to ask a reference laboratory to perform the analysis.

Firstly, all the biosafety measures required to work with risk II microorganisms were successfully implement at A4F's laboratory enabling all workers to understand the risks and measures required to perform not only *Vibrio* spp. analysis but also future experiments with other risk II microorganisms. Overall, during the process of implementing this method in a company with a limited background with bacteria, some challenges had to be overcome. Additionally to the fact that vibrio is a risk II microorganisms, which required special biosafety measures, protocols had to be developed for growth, maintenance and cryopreservation of bacteria. Furthermore, all the constraints within a company, either financial or availability of material or availability of the biosafety cabinet, had to be overcome during the implementation of the method.

Additionally, considering the resources available, the second goal was to use FISH probes provided by Biomode SA as a method to detect *Vibrio* spp. directly in microalgae cultures samples. We can conclude that Sudan Black B successfully inhibits the autofluorescence of microalgae and the limit of detection of this method is 10^6 CFU mL^{-1}. Therefore, it is possible to infer that if the microalgae culture is contaminated with *Vibrio* sp. with a concentration lower than 10^6 CFU mL^{-1}, the method is not able to detect it. *Vibrio* spp. have never been detected in microalgae cultures at A4F, possibly because, as previously showed in other studies, *Nannochloropsis* could suppress both quantitatively and qualitatively the growth of some *Vibrio* spp. (Taniguchi *et al.*, 2011). Considering this fact, even if present, the CFU mL^{-1} would be expected to remain low, close to zero CFU mL^{-1} of culture. Hence, an enrichment step, such as enrichment in ASPW would be required in order to evaluate the presence of *Vibrio* spp. but not its quantification. Together with the prerequisite of the additional steps to inhibit the microalgae autofluorescence, the method proved to be time-consuming and with a detection limit above the expected values for *Vibrio* spp. in the cultures. Hence, unless *Vibrio* spp. become a common contamination in cultures at A4F, and with values higher than 10^6 CFU mL^{-1}, routine analysis of the culture using FISH are not suitable. The detection of *Vibrio* spp. in the cultures would lead to the disposal of these contaminated cultures in order to avoid further costs in cultures that could not be commercialized. Thus, as future perspectives, probes could be developed with a different fluorochrome that can be distinguished from the autofluorescence of the sample (fluorescence in the red wavelengths spectrum). Even more, considering that when cells lose their viability the signal turns greenish/yellowish as seen in **Figure 6.4** in the appendix, these wavelengths range should also be avoided. Additionally, it should also be taken into consideration the fluorescence microscopy equipment available at A4F (**Table 2.2)**. Considering all the aspects above and the information available in the technical specifications data sheet (UIS2 fluorescence mirror units) by Olympus (n.d.), fluorescamine (excitation maximum: 390 nm; emission maximum: 460 nm) represents a possible fluorochrome used with the filter cube U-FVN.

Regarding the international standard method, ISO 21872-1:2017, until the final method was tested, several specific conclusions regarding the method itself were discussed along the experiments. Only the main conclusions are detailed below.

At first, it was possible to conclude that both CHROMagar™ and TCBS allow the growth of a wide range of false positive colonies with 1 mm or less. Considering that *V. cholerae* positive control presented colonies with only around 1 mm on TCBS, the size of typical colonies was neglected as a discriminatory factor and all green and yellow colonies on TCBS and mauve or blue colonies on CHROMagar™ proceeded to confirmation steps which resulted in a time-consuming method. But the fact is that there was a difference between the size of the colonies in the sample plates (1 mm or less) and the size expected (2 - 3 mm) on TCBS and the mauve and blue colonies on CHROMagar™ were only observed in agglomerates. These evidences, along with fact that *V. cholerae* positive control presented colonies similar to *V. vulnificus*, as expected, on CHROMagar™, could indicate that these colonies are not *Vibrio* spp., so further confirmation was required until these colonies could be discarded as *Vibrio* spp. For all the samples the official result of the analysis performed was that "Potentially enteropathogenic *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus* were not detected in 25 g of the paste according to the ISO 21872-1:2017". This conclusion is in agreement with the result previously obtained by the reference laboratory showing that these small false positives colonies were not *Vibrio* spp.

With the results later obtained from the spiked samples, we can conclude that typical colonies of the targeted species were identified on both media presenting the predictable dimensions according to ISO 21872-1:2017. After confirmation with PCR, it is possible to conclude that some strains can be inhibited on TCBS, resulting in smaller colonies. However, the fact that they grow to form typical colonies in the second selective medium allows their detection. Furthermore, the fact that, like in the positive control, *V. cholerae* originates the blue colonies with around 1 - 2 mm on CHROMagar™ confirms the higher efficacy of the CHROMagar™ medium to detect it and the importance of the complementarity between the two media as previously stated by other authors (Rosec *et al*., 2012). Concluding, even though a high number of false positive colonies is present, the difference in size between them and the positive controls along with the complementarity of both media allows to detect the three *Vibrio* spp. and to rule out false positives, including the colonies with less than 1 mm on TCBS.

Regarding the comparison of the two methods, direct PCR to the enrichment broths and the official method, the three species were not detected in any of the samples tested with both methods. With this and taking into account the negative result obtained by a reference laboratory, we can conclude the congruence of the results obtained. In the spiked samples only *V. cholerae* presented discrepancy. This species was detected on the mauve CHROMagar™ colonies for both spiked samples but only detected in the screening at 41.5 °C for sample ≈ 100 CFU by a faded band. This proves that no tested method is ideal and although, in previous studies, PCR directly to the enrichment broth showed to be more successful in detecting *V. parahaemolyticus* in at least double the number of samples than the plate culture method (Blanco-Abad *et al.*, 2009), this result indicates that PCR screening of enrichment broths is not, for itself, a reliable method to replace the ISO 21872-1:2017 method. As previously stated by different authors, there are different causes of inhibition during direct PCR with food or environmental samples (Blackstone *et al*., 2003; Kaufman *et al*., 2004). Some components of food and culture media, such as fats and proteins, have been reported to interfere or inhibit PCR (Rossen *et al*., 1992). Although, as future perspectives, systems could be used to purify the DNA to reduce inhibitions, these significantly increase time and cost of the analysis, which results in a serious constraint to include their use in routine analysis (Perry *et al*., 2007). Hence, more tests should be performed regarding this mater to infer the range of inhibitions that occur with this method, affecting the results obtained. Even more, considering that in the spiked samples *V. cholerae* was only detected at 41.5 °C, the screening should always be performed for both temperatures and not only for 37 °C.

Finally, the reference laboratory never performs the confirmation step, merely doing the previous steps to conclude the absence of *Vibrio* spp. This is in accordance with the fact that the colonies with 1 mm or less obtained on TCBS do not require additional confirmation and can be ruled out as *Vibrio* spp. Even considering that the analysis is done in an industrial scale production with three analysis together, the cost per analysis is approximately three times higher than the price provided by the reference laboratory. Hence, we can infer that it is not cost-effective to implement the method at A4F and the final conclusion is that it is preferable to ask a reference laboratory to perform the analysis. The main costs associated with this method are the human resources and the second selective medium. As future perspectives, even though CHROMagar™ was the cheapest selective differential medium for *Vibrio* spp. available by the company suppliers, another better cost-efficient medium for *Vibrio* spp. detection can be developed in the future. Even more, to date, the reference methods used to detect *Vibrio* rely mainly on microbiological techniques, via enrichment steps and isolation on culture media. Nevertheless, according to the emergence of hazards associated with the seafood coupled with *Vibrio* spp., studies on molecular methods have been increasingly developed in recent years (Bonnin-Jusserand *et al.*, 2019). Considering that this topic is constantly being improved in order to develop faster and more sensitive methods that can quickly be implemented, it is expected that new reference methods are developed in the future.

Finally, as previously mentioned, the absence of these *Vibrio* spp. is expected in the cultures. Therefore, the VBNC and dead cells topic, as well as quantitative methods, have not been discussed in-depth considering that these would only be crucial if *Vibrio* spp. was, in fact, detected in the cultures. If in the future that happens to be the case, these topics, including the ISO/NP TS 21872-2, still in proposal, dedicated to the quantitative determination in seafood of total and potentially enteropathogenic *V. parahaemolyticus* using nucleic acid hybridization, would have to be considered. The implementation of these methods would allow positive samples for *Vibrio* spp. to still be commercialized considering that the values were below the stipulated by the ISO/NP TS 21872-2. Even more, if *Vibrio* spp. become a common contamination, routine analysis must be implemented in order to avoid waste of resources in contaminated cultures. At that time, direct molecular methods such as the FISH of PCR directly to enrichment broths must be further tested to infer the best method for this purpose.

Chapter 5 - References

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Chapter 6 - Appendix

Table 6.1 – United Kingdom guidelines for pathogenic *V. parahaemolyticus* in ready-to-eat foods (FAO & WHO, 2017).

Result CFU/g	Risk category	Interpretation	Cause	Action	Laboratory specialist and reference tests
$>10^3$	High	UNSATISFACTORY Potentially injurious to health and/or unfit for human consumption	Strong evidence for poor processing	Immediate investigation of the food origin, review cooking and subsequent temperature and time controls. Take investigative samples of processed (cooked) food, raw food components (particularly marine products) and the food preparation environment.	Confirmation of identity; typing.
20 – $<10^3$	Moderate	UNSATISFACTORY	Little evidence for poor processing or cross-contamination	Risk will increase proportional to levels detected. Food may not become hazardous provided appropriate levels of control are applied. Consider taking investigative samples of processed (cooked) foods, raw food components (particularly marine products) and the food preparation environment.	Consider referral of isolates, particularly where associated with outbreak investigations.
<20	Low	SATISFACTORY		No further action required.	

Table 6.2 – United States of America standards for *Vibrio* spp. in seafood products (FAO & WHO, 2017).

Product	Requirement
Ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio cholerae* – absence of toxigenic O1 or O139 or non-O1 and non-O139 in a 25-gram sample
Ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio parahaemolyticus* – levels less than 1 x10^4 MPN/g (Kanagawa-positive or -negative)
Post-harvest processed clams, mussels, oysters, and whole and roe-on scallops, fresh or frozen, that make a label claim of "processed to reduce *Vibrio parahaemolyticus* to non-detectable levels"	*Vibrio parahaemolyticus* – levels less than 30/g (MPN)
Cooked ready-to-eat fishery products (minimal cooking by consumer)	*Vibrio vulnificus* – absence of organism[1]
Post-harvest processed clams, mussels, oysters, and whole and roe-on scallops, fresh or frozen, that make a label claim of "processed to reduce *Vibrio vulnificus* to non-detectable levels"	*Vibrio vulnificus* – levels less than 30/g (MPN)

Table 6.3 - List of the equipment used in this work and its respective model and manufacturer.

Equipment	Model – Manufacturer
Incubator	INCU-line - VWR
Precision scale (± 0.2 g)	NV 4101 - Ohaus
Analytical scale (± 0.0002 g)	PA114C - Ohaus
Microcentrifuge	5453 - Eppendorf
Microplate reader	SPECTROstar Nano - BMG LABTECH
Thermal cycler	Arktik thermal cycler - Thermo scientific
UV transilluminator	3UV, LMS-26 - Ultra-Violet Products
Microscope	BX53 - Olympus
Reflected fluorescence system	U-RFL-T - Olympus
Class II biological safety cabinet	Bio II Advance - Telstar

Table 6.4 - List of reagents used in this work and their respective supplier.

Reagent	Supplier
Sudan Black B	Scharlau
Sodium Chloride (NaCl)	Honeywell
Potassium chloride (KCl)	ChemLab
Monopotassium phosphate (KH_2PO_4)	Sigma
Sodium phosphate dibasic dihydrate ($Na_2HPO_42H_2O$)	Panreac
Formaldehyde (CH_2O)	Scharlau
Tris Base	Fisher Scientific
Triton-X-100	Sigma
TCBS Agar	Liofilchem and HIMEDIA
CHROMagar™ Vibrio plates	Frilabo
Agar powder	HIMEDIA
Yeast extract	OXOID
Tryptone	Liofilchem
Meat extract	HIMEDIA
Peptone	HIMEDIA
Tris-Acetate-EDTA (TAE) 50X solution	Fisher Scientific
Water for Molecular Biology	Nzytech
Agarose Tablets	Thermo Scientific
Green Safe Premium	Nzytech
Vibrio spp. primers	Nzytech
NZYTaq II 2x Green Master Mix 500 U	Nzytech

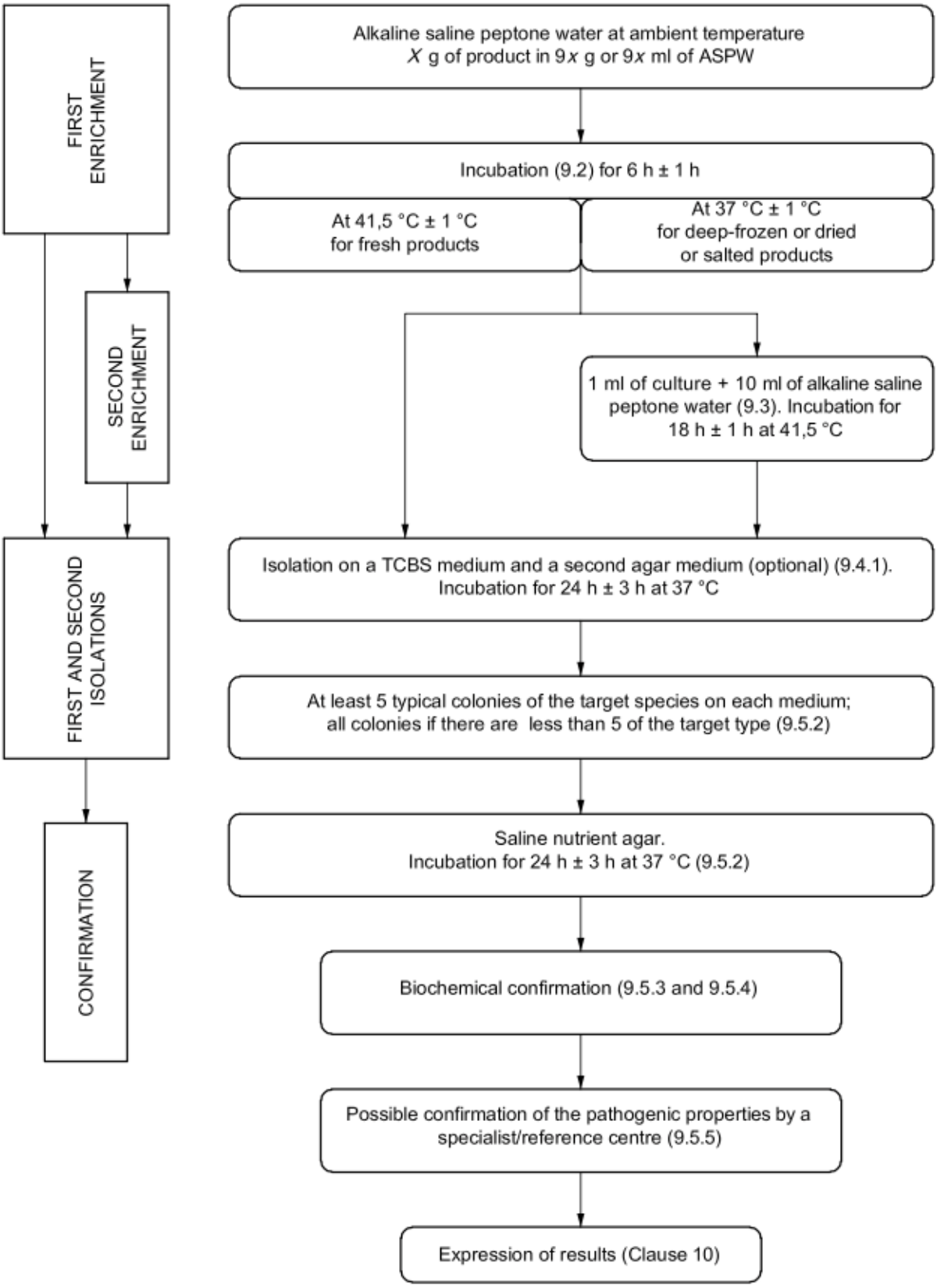

Figure 6.1 - Diagram of procedure according to the ISO/TS 21872-1:2007 (ISO, 2007).

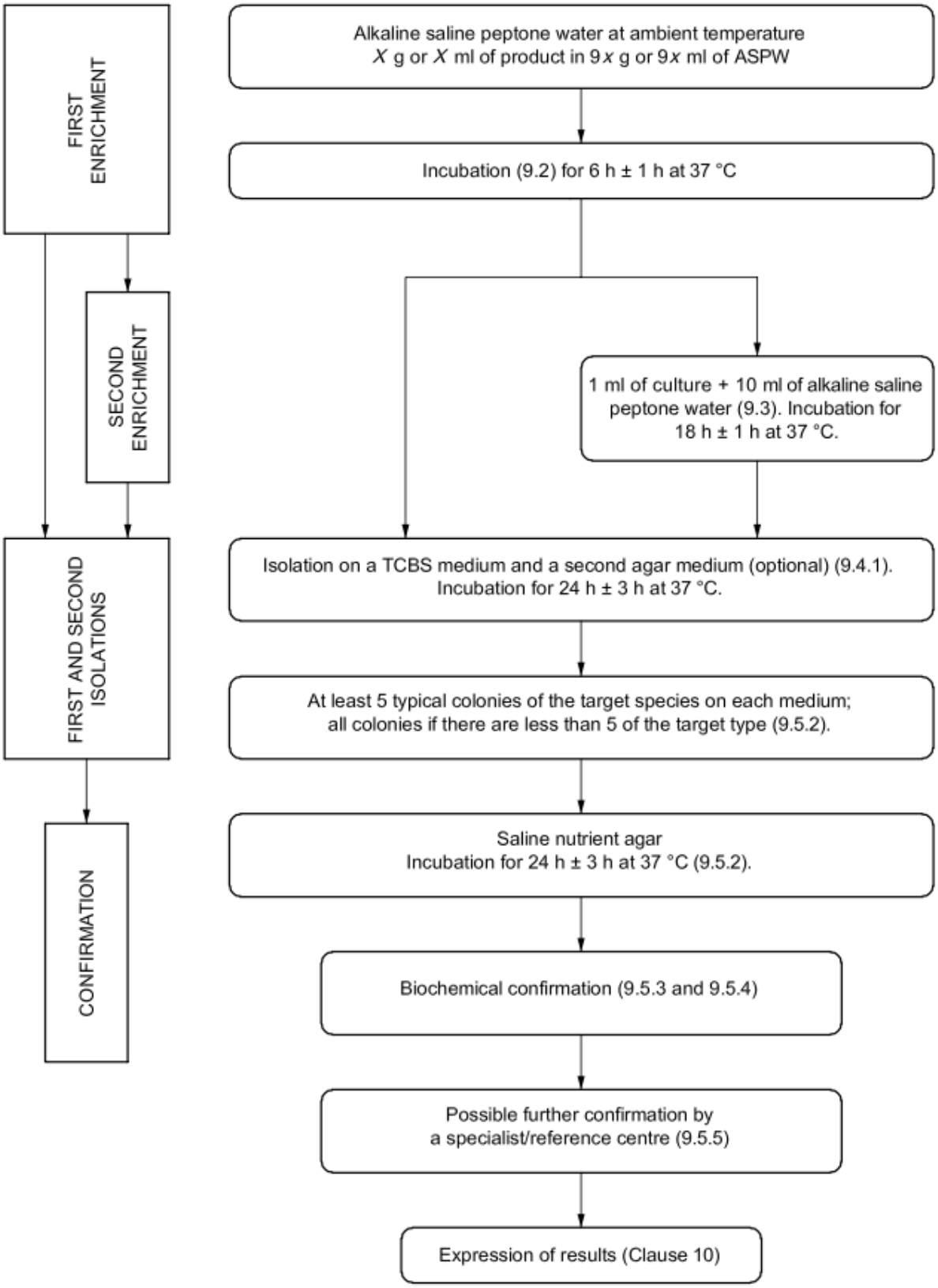

Figure 6.2 - Diagram of procedure according to the ISO/TS 21872-2:2007 (ISO, 2007).

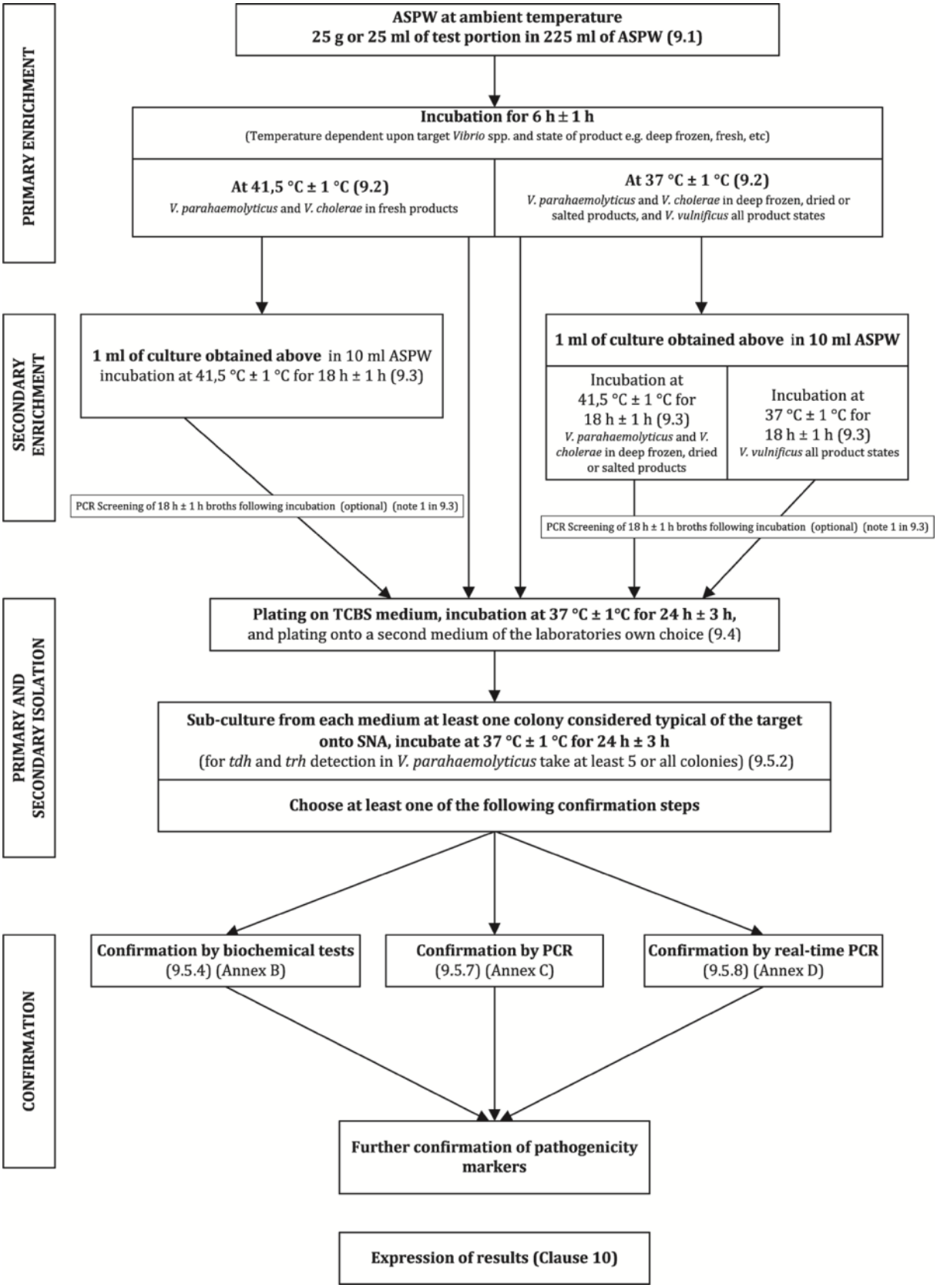

Figure 6.3 - Diagram of procedure according to the ISO/TS 21872-1:2017 (ISO, 2017).

Table 6.5 - Prices of each material required to perform the analysis until first and second enrichment.

Product	Price (€)	Price per unit (€)	Observations
1000 µL filter tips (8 · 96 units)	45.360	5.670 one box 0.059 one tip	
100 µL filter tips (10 · 96 units)	42.000	4.200 one box 0.044 one tip	
10 µL filter (10 · 96 units)	42.000	4.200 one box 0.044 one tip	
10 mL graduated pipettes (200 units)	28.000	0.140	
1 µL inoculating loops (2000 units)	40.800	0.021	
Cell scraper (100 units)	68.000	0.680	
1.5 mL micro-centrifuge tubes (500 units)	3.470	0.007	
15 mL falcon tubes (500 units)	39.100	0.078	
CHROMagar™ Vibrio (20 plates)	29.750	1.488 per plate	
T.C.B.S. Agar (500g)	40.750	0.182 per plate	89.2 g are added to 1 L of deionized, distilled water. Considering 25 mL are poured into each petri dish, 1 L gives a total of 40 plates. 500 g gives approximately 224 plates.
ASPW	Peptone - 0.071 per g Sodium chloride - 0.005 per g	1.520 per L	20 g Peptone, 20 g sodium chloride to 1 L of deionized, distilled water.
Sodium chloride (1000 g)	5.200	0.005 per g	
Peptone (500 g)	35.260	0.071 per g	
Petri Dishes (575 units)	39.470	0.068 per plate	
Salary of biology/biotechnology technician	1022.000 per month (The WageIndicator Foundation (2019)	6.083 per hour	Considering that 2018 had 252 working days, there is an average of 21 working days per month. A workday is considered to have 8 hours.

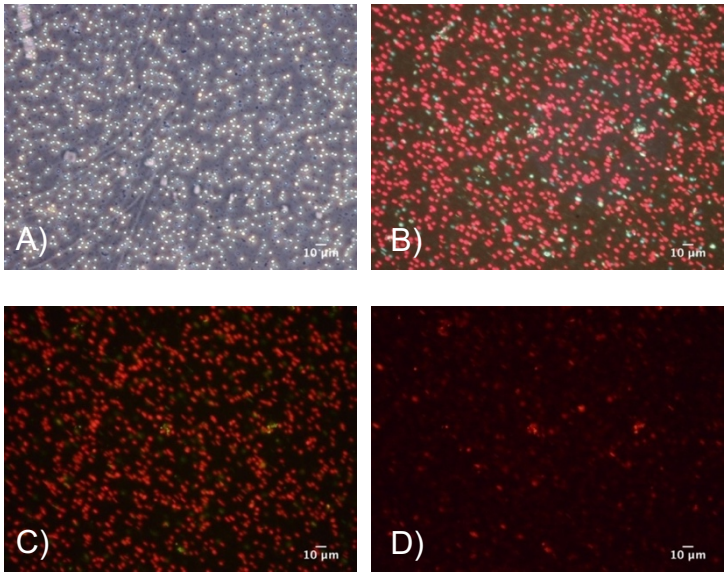

Figure 6.4 - *Nannochloropsis* sample under phase contrast (A), and fluorescence microscopy using filter cubes U-FVN (B), U-FBN (C) and U-FGNA (D).