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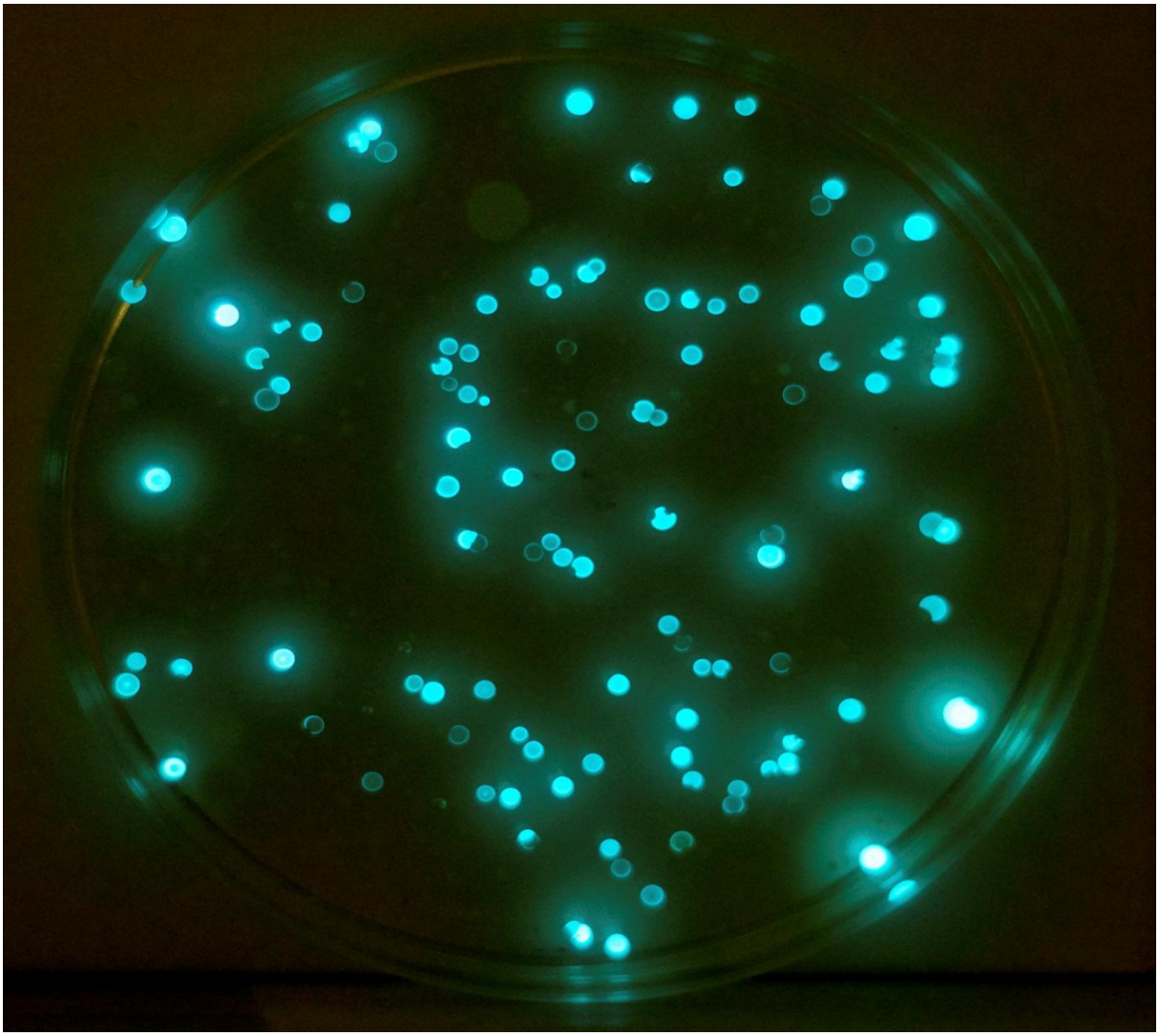

# Validation of Shelf-life Model designed for Cold Stored Foods

*Characterization of spoilage flora and initial contamination*

Master's thesis in Biotechnology

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Department of Chemical and Biological Engineering
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Cover:
[Luminescent *Photobacterium phosphoreum* cultivated on Long and Hammer´s medium at SIK, The Swedish Institute for Food and Biotechnology, Photo: Marie Blomqvist]

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# Abstract

Today large amounts of good quality cold stored foods are thrown away due to expiration of labeled static shelf life. In order to minimize waste and improve product safety there is a need to study the concept of a dynamic shelf life. One approach in this area is the three year Vinnova funded innovation project DYNAHMAT (in Swedish: Dynamiskt Hållbarhetsdatum för Minimerat Svinn), operated by Lund University together with the Swedish Institute for Food and Biotechnology (SIK) and Malmö University. The project goal is to develop intelligent logistics- and packaging systems that communicate and predict quality and product safety of cold stored food in real-time. Since shelf-life of such products most often is limited by microbial action models describing growth of the specific organism responsible for spoilage must be applied. For the models to predict time to spoilage an input variable representing the initial amount of the specific spoilage organism (SSO) is needed.

The aim of this thesis work is to determine the initial concentration of the SSO responsible for spoilage of modified atmosphere packed (MAP) cod, previously identified as *Photobacterium phosphoreum*. With the starting concentration known, the purpose is furthermore to validate an existing shelf-life predictive model that is ought to be applied in the DYNAHMAT project. Moreover, the intention is to identify and characterize the general spoilage flora of MAP cod.

Initial cultivation and storage studies revealed aerobically incubated Long and Hammer plates as the most appropriate method for determination of the initial amount of the SSO. Partial 16s rRNA gene sequencing identified glass-like colonies growing on these plates as members of the *Photobacterium* family, but was unfortunately not enough to distinguish *P. phosphoreum* from other closely related *Photobacterium* species.

Based on the assumption that observed glass-like colonies are *P. phosphoreum*, performed long term storage study resulted in an initial concentration of 2.15 log (cfu/g) for fish fillets packed in an modified atmosphere immediately after filleting. Shelf-life model validation revealed that the model is able to accurately illustrate growth of *P. phosphoreum* and based on existing sensory rejection evaluation hence predict shelf-life of MA-packed cod.

Future studies including multi-locus gene analysis need to be performed in order to reliably conclude that glass-like colonies aerobically cultivated on Long and Hammer plates exclusively belong to the specie *P. phosphoreum*. If the outcome of these studies reveals a mixed *Photobacterium* flora the respective species must be inoculated into fresh cod and spoilage development must be studied in order to confirm *P. phosphoreum* as the SSO of MA-packed cod and exclude other species contribution to fish spoilage. Last but not least, the performed long term storage study needs to be repeated in order to find a truly representative initial concentration of the SSO that hence can be used to accurately predict shelf-life of MA-packed cod in the DYNAHMAT project.



Keywords: dynamic shelf-life, predictive microbiology, MA-packed cod, *Photobacterium Phosphoreum.*

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# 1. Introduction

## 1.1 Background

Today large amounts of good quality cold stored foods are thrown away due to expiration of labeled shelf life. In Sweden and other parts of Europe as much as a third of all produced/imported food is discarded (WRAP 2008) and the waste of cold stored foods take place both at the consumer level and in the food distribution chain (Karlberg & Klevås 2002). According to WRAP (2008) and Rahelu (2009) approximately 60 % of all food that is thrown away could have been eaten if it was possible to determine quality in real time. Furthermore, the usage of a predetermined static shelf life is not always reliable when it comes to food quality assurance. This since each food item in its distribution chain is subjected to specific, often varying storing conditions that are impossible for the shelf life labeling producer to predict.

In order to minimize waste of good quality foods and improve product safety there is a need to study the concept of a dynamic shelf life. One approach in this area is the three year Vinnova funded innovation project DYNAHMAT (in Swedish: Dynamiskt Hållbarhetsdatum för Minimerat Svinn), operated by Lund University together with the Swedish Institute for Food and Biotechnology (SIK) and Malmö University. The project goal is to develop intelligent logistics- and packaging systems that along the food chain communicate and predict quality and product safety of cold stored food in real-time. To succeed, the plan is to identify, evaluate and develop sensors able to measure parameters effecting food quality and then combine these with predictive models for determination of shelf life and safety. The sensors and models can then be integrated with distributed information systems and communication tools which will enable the delivery of a dynamic shelf life. One possible approach is the attachment of a temperature registration sensor directly to the food product and the subsequent utilization of e.g. a mobile phone application for the prediction and delivery of shelf-life.

Included in the DYNAHMAT project are three cold stored food products; fresh cod packed in modified atmosphere (MA), cottage cheese and a MA-packed ready to eat processed meat product. Since shelf-life of these products most often are limited by microbial action models describing growth of the specific organism responsible for spoilage must be applied. Such mathematical models have been developed during the last decades and a few are today available for shelf-life prediction. However, for the models to predict time to spoilage they, except for the food storage temperature registered by the sensor, need an input variable representing the initial amount of the specific spoilage organism (SSO). Such SSO starting concentrations must therefore be experimentally determined and the predictive models need to be validated against experimental data before they can be applied in future DYNAHMAT solutions.

## 1.2 Aim

The aim of this thesis work is to determine the initial concentration of the specific spoilage organism (SSO) responsible for spoilage of modified atmosphere packed (MAP) cod, previously identified as *Photobacterium phosphoreum.* With the starting concentration known, the purpose is furthermore to validate an existing shelf-life predictive model that is ought to be applied in the

DYNAHMAT project. Moreover, the general spoilage flora of MAP cod will be characterized and identified in order to confirm the spoilage flora development reported in literature.

### 1.3 Limitations

Although there are three cold stored food products included in the DYNAHMAT project fresh cod packed in modified atmosphere is the only one included in this thesis work due to a limited timeframe. In addition, the restricted availability of time only allows traditional plating techniques to be applied for the SSO initial concentration determination. Furthermore, the validation procedure will be limited to growth of the SSO and hence no sensory rejection validation will be performed.

## 2. Theory

### 2.1 Spoilage of modified atmosphere-packed cod

Fresh fish has due to high water activity ($a_w$), neutral pH and presence of autolytic enzymes limited shelf-life (Sivertsvik et al., 2002). Spoilage is according to Sivertsvik et al. among others most often caused by microbial action and various microorganisms are naturally present on all outer fish surfaces (i.e. skin and gills) as well as inside the fish intestines. Although the fish muscle itself is sterile, it easily becomes infected by the surface and intestinal bacteria during slaughtering and processing. The microbial spoilage of fresh fish depends on both intrinsic (e.g. pH and fish quality) and extrinsic factors such as storage temperature, processing and packaging atmosphere (Huss et al., 1997). Microbial fish spoilage is often characterized by undesirable changes in appearance, flavour, texture and odour resulting in loss of food quality (Sperber & Doyle, 2009). Odorous compounds normally produced towards the end of fish shelf-life due to bacterial growth are volatile fatty acids, ammonia, low molecular weight sulphur compounds such as hydrogen sulphide ($H_2S$) and trimethylamine (TMA) (Sivertsvik et al., 2002). TMA is recognized as the characteristic “fishy” odour of spoiled fish and are at chilled temperatures produced by bacterial reduction of trimethylamine-oxide (TMAO) catalyzed by the bacterial enzyme TMA oxidase (Ashie et al., 1996). Many spoilage bacteria can according to Sivertsvik et al. (2002) also produce TMA when growing in oxygen limited environments by utilizing TMAO as a terminal hydrogen acceptor.

The aim of modified atmosphere packaging is replacement of air with another fixed gas mixture that often consists of $CO_2$ and/or $N_2$. $N_2$ is an inert gas that is used for oxygen removal, thus preventing growth of aerobic bacteria, whereas $CO_2$ exerts bacteriostatic properties that inhibit growth of many spoilage bacteria. The mode of inhibitory action is not completely known but $CO_2$ is thought to create a drop in pH and at the same time decrease enzymatic and microbial activities (Sivertsvik et al., 2002). Devlieghere et al. (1998) have demonstrated that $CO_2$ needs to dissolve into the product in order to inhibit bacterial growth. Carbon dioxide is highly soluble in water and fat and the solubility increases with decreased temperature, i.e. the inhibitory effectiveness is greatly influenced by storage temperature and the benefits of MAP may be lost without proper storage control (Sivertsvik et al., 2002). Furthermore, Sivertsvik et al. stresses that in order to reach an appropriate bacterial inhibition and at the same time prevent package collapse due to $CO_2$- solubility the volume of gas should be at least two to three times the volume of fish product.

During fish storage the bacterial microflora may develop in certain directions, i.e. some bacterial species may grow more than others depending on surrounding conditions. Although a spoiled fish product contains a mixture of bacterial species there is often a specific bacterial group that is responsible for the important chemical changes coupled to fish spoilage (Huss et al., 1997). This specific bacterial specimen is usually referred to as an SSO (Specific Spoilage Organism) and even though the knowledge of SSOs of different fishes are still limited (Sivertsvik et al., 2002) the SSO of both aerobically stored and MA-packed cod have been identified. Already in 1987 studies performed by Gram et al. revealed that *Shewanella putrefaciens* is the main spoilage bacterium of cod stored aerobically at chilled temperatures. The genus *Shewanella* belongs to the Alteromonadaceae family and comprises Gram negative and oxidase positive straight or curved rods that are facultatively anaerobic (Bowman, 2005). The specie *S. putrefaciens* produces intensive and unpleasant off-odours, reduces TMAO to TMA and is especially well-known for its ability to produce $H_2S$, with a smell strongly reminiscent of rotten eggs (Sivertsvik et al., 2002). In MAP cod *S. putrefaciens* is however strongly inhibited by $CO_2$ and the organism responsible for spoilage has been identified as *P. phosphoreum* (Dalgaard et al. 1993; Dalgaard 1995a).

### *2.1.1 Photobacterium phosphoreum*

*P. phosphoreum* is taxonomically classified as a Gammaproteobacteria and belongs to the family Vibrionaceae. It is a Gram negative, motile, facultative anaerobic and chemoorganotrophic bacterium that has both a respiratory and a fermentative type of metabolism. The morphology consists of plump, straight rods (some coccobacillary) and the bacterium is oxidase negative and motile by means of flagella (Thyssen & Ollevier, 2005). *P. phosphoreum* are psychrotrophic and widespread in the marine environment, where the most common habitat is in the intestines of marine fishes (Dalgaard et al., 1997a). It is able to grow at 4°C but not at temperatures above 35°C (optimum growth occurs between 20-25°C) and sodium ions are required for growth (Thyssen & Ollevier, 2005). Some strains of *P. phosphoreum* are bioluminescent and are thus able to emit a blue-green light. According to Thompson et al. (2004) the bacteria can live in symbiotic associations with marine fishes, where they colonize light organs of the host and via emission of light play a role in communication, prey attraction and predator avoidance. The bioluminescence is caused by an oxidation reaction that is catalyzed by the enzyme luciferase. The reaction involves oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde by molecular oxygen resulting in the formation of light (Thyssen & Ollevier, 2005):

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + light$$

The fact that *P. phosphoreum* has been identified as the SSO of MA-packed cod is not surprising since it has been shown to be highly resistant to $CO_2$ and the growth rate of the bacterium is increased under anaerobic conditions (Dalgaard, 1995b). Spoiled MAP cod is opposed to aerobically stored cod characterized by high levels of TMA, resulting in fishy and ammonia-like off-odours and sour off-flavours (Dalgaard, 1993). This observation can be explained by the fact that *P. phosphoreum* reduces TMAO to TMA at 10-100 times the amount per cell compared to *S. putrefaciens* and produces only very small amounts of the putrid $H_2S$ (Dalgaard, 1995a). The high TMA-producing ability can according to Dalgaard (1995a) probably be due to the large cell size (5µm in diameter) observed for *P. phosphoreum*.

## 2.2 Predictive microbial shelf-life models

### 2.2.1 Bacterial growth

In order to predict shelf-life within the food microbiology field bacterial growth needs to be studied and modeled. Predictive microbiology involves the development of mathematical models that can be used to describe the behavior of microorganisms under different physical or chemical conditions, such as for example temperature. Bacterial growth is characterized by more or less distinct subsequent phases that together form a sigmoidal curve (Zwietering et al., 1990), see Figure 1. Before growth can occur bacteria may need some time to adjust to ambient conditions and the growth curve therefore often consists of an initial part where the growth rate is close to zero, represented by the lag phase (Todar, 2012). The growth then accelerates to a maximal value within the exponential phase before a stationary phase is reached where the growth rate again approaches zero. After some additional time the number of bacteria starts to decline, representing the so called death phase. By defining the growth curve as the logarithm of the number of bacteria plotted against time the maximum specific growth rate ($\mu_{max}$) is given by the slope of the line when bacteria grows exponentially (Zwietering et al., 1990).

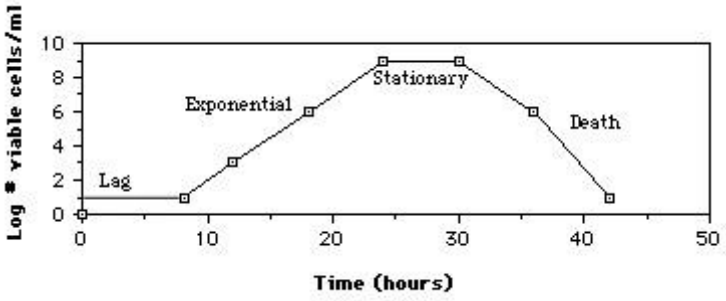


**Figure 1. A typical bacteria growth curve obtained by plotting the logarithm of the number of viable bacteria cells against time (Todar, 2012).**

### 2.2.2 Microbial model for shelf-life prediction of MAP cod

To be able to predict shelf-life of cod fillets packed in modified atmospheres with mixtures of $CO_2$ and $N_2$ Dalgaard et al. (1997b) applied an iterative approach to develop a microbial model based on the SSO *P. phosphoreum*. The iterative approach was divided into three separate parts; initial studies with naturally contaminated cod to determine spoilage domains and growth kinetics of the SSO, mathematical modelling of data including quantification of extrinsic and intrinsic parameter effects and finally model validation by comparison against data obtained from storage experiments. The work resulted in a well-functioning spoilage model that according to Dalgaard et al. (1997b) can be used to accurately predict shelf-life of MAP cod based on initial numbers of *P. phosphoreum*, storage temperature and the level of $CO_2$ in the modified atmosphere.

In order to determine the concentration of *P. phosphoreum* ($N_t$, in log cfu/g) at a specific time (t) a log-transformed three- parameter logistic model is used as a primary growth model,

where $N_0$ is the initial concentration of *P. phosphoreum* (in cfu/g), $N_{max}$ is the maximum concentration (in cfu/g) and $\mu_{max}$ is the specific growth rate (in $h^{-1}$):

$$\log(N_t) = \log\left(\frac{N_{max}}{1+\left(\frac{N_{max}}{N_0}-1\right)\times\exp(-\mu_{max}\times t)}\right)$$

Dalgaard et al. (1997a) determined that sensory rejection of MAP cod and thus end of shelf-life on average can be observed four generation times ($t_{gen} = \ln(2)/\mu_{max}$) after the inflection point of the logistic growth curve is reached. By using a $N_{max}$ value of 7.9±0.4 log cfu/g time to spoilage can thus be calculated from the initial concentration ($N_0$) and maximum specific growth rate ($\mu_{max}$) using the following shelf-life criterion:

$$Shelf-life = \frac{4\times\ln(2)+\ln[(N_{max}/N_0)-1]}{\mu_{max}}$$

The maximum specific growth rate ($\mu_{max}$) depends on storage temperature as well as the amount of $CO_2$ and a secondary quadratic polynomial growth model can be utilized to calculate its value:

$$\sqrt{\mu_{max}} = 0.29 + 0.032\times T - 1.6\times10^{-3}\times\%CO_2 - 9\times10^{-5}\times T\times\%CO_2 + 9\times10^{-6}\times(\%CO_2)^2$$

where T is storage temperature in °C and $\%CO_2$ is equal to the equilibrium concentration of carbon dioxide obtained after $CO_2$ has dissolved into the fish flesh.

#### *2.2.2.1 Determination of the initial amount of P. phosphoreum in cod*

To be able to apply the predictive *P. phosphoreum* shelf-life model for MA-packed cod presented in section 2.2.2 in the DYNAHMAT project, an initial concentration ($N_0$) of the specific spoilage organism needs to be experimentally determined. A couple of methods to determine the number of *P. phosphoreum* in fresh fish have been presented in the scientific literature over the past three decades. In general, there exist three distinct techniques; a real-time PCR method (Macé et al., 2013), a conductance method (Dalgaard et al., 1996) and various traditional cultivations methods utilizing culture plates (Dalgaard, 1995a; Dalgaard et al., 1997a; NMKL Method, 2006). Since the timeframe of this project is restricted only one concentration determining method can be applied and the traditional cultivation option was found as the most appropriate choice.

There are today no completely *P. phosphoreum* selective cultivation medium available (Macé et al., 2013), but Iron Agar and Long and Hammer´s medium both containing 1 % NaCl have been suggested as appropriate alternatives (Dalgaard, 1995a & Dalgaard et al., 1997a). By utilizing spread plates of the suggested media *P. phosphoreum* can be distinguished and isolated as glass-like colonies. $H_2S$ producing *S. putrefaciens* can on the other hand be determined as black colonies on Iron Agar pour plates due to precipitation of iron sulphide (FeS) (Dalgaard, 1995a; NMKL Method, 2006).

Although traditional plating is a reliable method for *P. phosphoreum* cultivation the usability for direct determination of the initial number of bacteria may be limited. The initial bacterial flora often consists of a mixture of various species and it seems probable that the starting concentration of *P. phosphoreum* lies under the detection limit. However, by utilizing the traditional plating technique the growth curve of *P. phosphoreum* can be constructed. Linear regression of the exponential phase followed by extrapolation can then be utilized in order to find the sought starting concentration (i.e. the point where the straight line intercepts with the y-axis).

# 3. Materials and methods

## 3.1 Initial studies for growth media and cultivation technique selection

In order to characterize the natural cod bacterial flora and try to find an appropriate *P. phosphoreum* cultivation method, different media and cultivation techniques were evaluated by microbial analysis. Identification tests followed by PCR and sequencing were then performed to confirm the identities of isolated bacteria colonies.

### 3.1.1 Media and cultivation techniques

Media and cultivation methods included in the initial studies are presented in Table 1. Since *P. phosphoreum* (in contrast to *S. putrefaciens*) is highly $CO_2$-resistant (Dalgaard, 1995b) two cultivation methods involving storage in an atmosphere of 100% $CO_2$ were included.

**Table 1. A summary of growth media and cultivation conditions used in initial studies.**

| Cultivation method | Medium | Plating | Temperature | Time |
|---|---|---|---|---|
| **LH 1% NaCl Aer** | NMKL Method No. 184 (2006) | Spread | 15°C | 6d |
| **LH 1% NaCl $CO_2$** | NMKL Method No. 184 (2006) | Spread | 15°C | 6d |
| **IA 0.5% NaCl Aer** | Oxoid agar CM964 | Pour | 25°C | 3d |
| **IA 1% NaCl Aer** | Oxoid agar CM964 + 5g NaCl/l | Spread | 5°C | 14d |
| **IA 1% NaCl $CO_2$** | Oxoid agar CM964 + 5g NaCl/l | Spread | 25°C | 6d |

LH: Long and Hammer´s agar, IA: Iron Agar, Aer: Aerobic storage of plates, $CO_2$: Plates stored in 100% $CO_2$
Spread: 0.1ml sample was evenly spread on top of the agar
Pour: 1ml sample was mixed with 10-15ml agar and a thin layer of IA was spread on top of the solidified first agar layer

### 3.1.2 Microbial analysis and CFU determination

Fillets from two individual cod fishes (A and B) caught in the northeast Atlantic were bought in a local store and transported to SIK within 20 minutes. Microbial analysis was performed immediately at day 0 and after 3 days of storage at 4°C in modified atmosphere (50 % $CO_2$/50 % $N_2$ using a Henkovac vacuum packer from HFE Vacuum Systems, the Netherlands). Fish samples

were prepared by tenfold dilution of 10g of cod in chilled peptone water (PW) followed by homogenization for 60 s in a Stomacher. The homogenates were further diluted in PW and spread and pour plates were prepared according to Table 1. LH 1% NaCl $CO_2$ and IA 1% NaCl $CO_2$ were placed in plastic bags and packed in 100% $CO_2$. After incubation all spread plates were inspected for luminous colonies (performed in a dark room) and standard plate count was utilized in order to determine the number of each phenotypic colony forming unit (cfu) as well as total viable count (TVC).

### 3.1.3 Identification tests

In order to study observed colonies closer a total of 32 individual colonies were selected from LH 1% NaCl Aer, LH 1% NaCl $CO_2$ and IA 1% NaCl $CO_2$ from day 0 (both fillet A and B) and isolated on LH 1% NaCl during aerobic conditions. The selection procedure was designed so that all various phenotypic types of colonies were included in the identification. All isolated colonies were examined for macroscopic morphology and ability to produce cytochrome C oxidases, using Reagent Dropps (BD Oxidase, United States) whereas a total of 13 colonies were further investigated by Gram staining and microscopy.

### 3.1.4 PCR and sequencing

To confirm the identities of phenotypically different colonies originally picked from LH 1% NaCl Aer and LH 1% NaCl $CO_2$ seven out of the 13 colonies that were studied in more detail in the identification procedure were selected for PCR and subsequent sequencing. Moreover, to be able to specifically identify glass-like colonies growing on LH 1% NaCl Aer and LH 1% NaCl $CO_2$ another 42 colonies were randomly picked, isolated and subjected to PCR and sequencing. The 42 colonies consisted of 32 with origin from LH 1% NaCl $CO_2$ (eight colonies from each cod and storage day) whereas the remaining ten glass-like colonies were picked from LH 1% NaCl Aer from storage day 3 (five from each cod). No glass-like colonies from LH 1% NaCl Aer storage day 0 were included due to difficulty in distinguishing these from other colonies. However, at the day of isolation two individual colonies with distinct characteristics had appeared on LH 1% NaCl Aer from storage day 0 and these were therefore also subjected to PCR and sequencing. In other words, a total of 51 individual colonies were included in the PCR and sequencing procedure.

PCR was performed using primer F8 (5'- AGA GTT TGA TCC TGG CTC AG-3') and 926R (5'-CCG TCA ATT CCT TT R AGT TT -3') for amplification of a 918bp fragment of the bacterial 16S rRNA. Amplification was carried out in a 25µl reaction mixture containing 1x iProof High-Fidelity Master Mix (Bio-Rad Laboratories, United States), 0.5µM of each primer, 2 µl pre-boiled DNA template (made by dissolving colonies in 0.5ml deionized and sterilized water) and sterile deionized water to reach a final volume of 25µl (due to cases with week PCR products some colonies were directly dissolved in the PCR mixture without pre-boiling). A negative control (sample without DNA template) was included in all experiments for contamination detection. The PCR amplification was carried out with a thermal cycler machine (Bio Rad Laboratories) under the following conditions: initial denaturation (3 min, 98°C) followed by 35 cycles of denaturation (10 s, 98°C), annealing (30 s, 55°C) and extension (1 min, 72°C) and a final elongation step (10 min, 72°C).

The amplified products were visualized by electrophoresis with a PowerPac Basic (Bio-Rad Laboratories, United States) along with a Fermenta Fastruler DNA ladder of Low Range (Thermo

Scientific, United States) using a 1.5% (w/v) agarose gel. 4 µl PCR products together with 1 µl Gel Red Loading Dye solution (1µl GelRed Nucleic acid stain 10,000x in DMSO from Biotium, United States and 100µl Nucleic acid sample loading buffer 5x from Bio-Rad, United States) were added to each well. The amplified and visualized PCR products were according to the company's recommendations send to Macrogen Europe (Amsterdam, the Netherlands) for DNA sequencing. Bacteria were identified by aligning obtained sequences using the Basic Local Alignment Search Tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (RDP) Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp), respectively.

## 3.2 Long term storage studies

To be able to construct growth curves for the SSO *P. phosphoreum* three separate long term storage studies were performed using appropriate cultivation methods found in initial studies, see Table 2. In all studies fillets from cod newly caught in the northeast Atlantic were delivered to SIK kept on ice (for study number three fish fillets were after filleting at the fish distributor immediately packed in a modified atmosphere (50% $CO_2$/50% $N_2$) and hence arrived MA-packed). The fillets were aseptically cut into separate pieces and placed in bags made by plastic film delivered from the fish distributor. In study number three all fish pieces were mixed in a plastic bag to obtain an evenly distributed bacterial flora prior to placement in individual packages. Microbial analysis was immediately performed as described in section 3.1.2. Remaining fish pieces were packed in modified atmosphere (50 % $CO_2$/50 % $N_2$ with a fish to gas volume ratio of 1:2-3 using a HenkoVac vacuum packer from HFE Vacuum Systems, the Netherlands) and stored at 2°C or 4°C. The microbial analysis procedure was repeated during different time intervals using fish packages containing <1% $O_2$ (the low oxygen levels and presence of carbon dioxide was confirmed using a CheckMate II Gas analyzer from PBI Dansensor, Denmark). A limited sensory analysis was also performed and included smell and texture observations. Plates belonging to LH 1% NaCl $CO_2$ were packed in plastic bags with 100% $CO_2$ and all plates were incubated according to Table 1. After incubation the carbon dioxide level for LH 1% NaCl $CO_2$ was controlled and all plates were inspected for bacterial growth.

**Table 2. A summary of details for the three long term storage studies performed.**

| | Media | Fillets/Replicates | Storage temp | Days of analysis* |
|---|---|---|---|---|
| **Study number 1** | LH 1% NaCl Aer<br>LH 1% NaCl $CO_2$<br>IA 0.5% NaCl Aer | Four fillets (A,B,C,D) | 2°C and 4°C | 0,1,4,6,8,11,13,15 |
| **Study number 2** | LH 1% NaCl Aer | Five fillets (A,B,C,D,E) | 4°C | 0,3,4,5,6,7,10 |
| **Study number 3** | LH 1% NaCl Aer | Five biological replicates | 4°C | 1,2,3,4,5,6,7,9** |

*Day of MA-packing designated as day 0

**Analysis performed two times a day except for day 9

# 4. Results and discussion

## 4.1 Initial studies for growth media and cultivation technique selection

### 4.1.1 Microbial analysis and CFU determination

Results from microbial analysis and CFU determination are summarized in Table 3 and 4, respectively. Luminescence was observed from some glass-like colonies on LH and IA spread plates incubated aerobically, indicating that these might be strains of *P. phosphoreum*. Furthermore, the number of luminescent colonies increased from day 0 to day 3. As expected, no luminescent colonies were observed on plates incubated in $CO_2$ since oxygen is required (see section 2.1.1).

As can be seen in Table 4, reported numbers of cfu are regardless of cultivation technique in the same region for fish fillets A and B. Small glass-like colonies were observed on all spread plates, see Table 3. However, LH 1% NaCl $CO_2$ was the only cultivation technique that displayed a completely uniform bacterial flora, consisting of only glass-like colonies. Regarding LH plates incubated aerobically, the numbers of glass-like colonies reported for day 0 are unsure since it turned out to be difficult to distinguish glass-like colonies from the competing bacterial flora. Nevertheless, the number of glass-like colonies reported for LH plates incubated aerobically corresponds fairly well to the TVC observed for LH plates incubated in $CO_2$. This observation supports the suspicion that $CO_2$ incubation inhibits all bacteria other than *P. phosphoreum* and reinforces the probability that glass-like colonies indeed are *P. phosphoreum*.

Common for both LH cultivation techniques are the increase of glass-like colonies from day 0 to day 3, which in combination with the fact that the same trend was observed for luminescent colonies further supports that glass-like colonies are *P. phosphoreum*. An increase of $H_2S$ producing black colonies could also be observed from IA pour plates, yet to a smaller extend compared to growth of glass-like colonies on LH. Regarding IA spread plates incubated aerobically the number of glass-like colonies correspond very well to the numbers found on aerobically and $CO_2$ incubated LH plates, indicating that these colonies probably represents the same bacterial species. However, since a much more complex bacteria flora was observed on these plates and the cultivation method demands a much longer incubation period (14 days compared to 6 days), the LH-techniques seem like better cultivation options. The IA $CO_2$ spread plate technique also seems like a less reliable method since it was really hard to distinguish between white and glass-like colonies. As a result only the TVC are included for IA $CO_2$ in the results presented in Table 4.

Table 3. Macroscopic characteristics of colonies observed in initial studies.

| | LH 1% NaCl Aer Spread | LH 1% NaCl $CO_2$ Spread | IA 0,5% NaCl Aer Pour | IA 1% NaCl Aer Spread | IA 1% NaCl $CO_2$ Spread |
|---|---|---|---|---|---|
| **Bacterial Flora** | Large pink<br>Large white<br>Small glass-like | Small glass-like | Small black<br>Small white | Large white<br>Large pink<br>Small glass-like<br>Black<br>Yellow<br>Intestine-like | Small white<br>Small glass-like |

Table 4. Number of colony forming units observed for the different cultivation techniques included in initial studies.

| | | Day 0 | | Day 3 | |
|---|---|---|---|---|---|
| | | *A* | *B* | *A* | *B* |
| **LH 1% NaCl Aer Spread** | Glass-like colonies (log cfu/g) | 6.18 | 5.57 | 8.15 | 8.01 |
| | Total viable count (log cfu/g) | 6.44 | 6.18 | 8.17 | 8.02 |
| **LH 1% NaCl $CO_2$ Spread** | Total viable count (log cfu/g) | 6.16 | 5.53 | 7.58 | 7.78 |
| **IA 0,5% NaCl Aer Pour** | Black colonies (log cfu/g) | 5.43 | 5.00 | 6.27 | 6.06 |
| | Total viable count (log cfu/g) | 6.21 | 6.03 | 6.46 | 6.21 |
| **IA 1% NaCl Aer Spread** | Glass-like colonies (log cfu/g) | 6.21 | 5.65 | 8.11 | 7.95 |
| | Total viable count (log cfu/g) | 6.45 | 6.20 | 8.12 | 7.96 |
| **IA 1% NaCl $CO_2$ Spread** | Total viable count (log cfu/g) | 5.27 | 4.89 | 6.40 | 6.74 |

### 4.1.2 Identification tests

Since microbial analysis revealed that both LH cultivation methods and to some extend also the IA 1% NaCl $CO_2$ technique could be appropriate cultivation techniques to study the growth of *P. phosphoreum,* tests were performed to identify isolated bacteria in question. An overview of test results can be found in Table 5. Unlike IA plates incubated in $CO_2$ both LH cultivation techniques displayed uniform results for each distinct macroscopic morphology tested. The small white colonies on IA turned out to consist of both gram positive and gram negative bacteria whereas the small glass-like ones appeared as oxidase positive large pink colonies when isolated on LH 1% NaCl Aer. This fact means that the glass-like colonies found on IA $CO_2$ cannot be *P. phosphoreum* (which is oxidase negative) and that most probably only a fraction of the white colonies belong to this specie. As a result, the IA cultivation technique could be ruled out for further studies.

Regarding test results for LH incubated aerobically it can be concluded that neither large pink nor large white colonies belong to the *P. phosphoreum* specie. Moreover, all test results for small glass-like colonies strengthens the suspicion that these are *P. phosphoreum,* regardless of LH technique.

Table 5. Results from identification tests performed on selected colonies from day 0.

| | Macroscopic morphology | Oxidase test | Gram staining | Microscopic morphology |
|---|---|---|---|---|
| **LH 1% NaCl Aer** | Large pink colonies | Positive | G- | Small/thin elongated straight rods |
| | Large white colonies | Positive | G- | Small, slightly thicker straight rods |
| | Small glass-like colonies | Negative | G- | Mixture of straight rods and coccobacilli |
| **LH 1% NaCl $CO_2$** | Small glass-like colonies | Negative | G- | Mixture of straight rods and coccobacilli |
| **IA 1% NaCl $CO_2$** | Small white colonies | Negative | G-/G+ | Mixture of straight rods and coccobacilli/straight rods |
| | Small glass-like colonies | Positive | G- | Small/thin elongated straight rods |

### 4.1.3 PCR and sequencing

PCR and sequencing were performed in order to confirm the identities of colonies with different macroscopic morphology found on LH Aer and LH $CO_2$. Results from alignment of obtained sequences using BLAST and RDP Classifier are displayed in Table 6. As can be seen, large pink and large white colonies were regardless of cultivation technique respectively identified as members of the *Shewanella* and *Pseudomonas* family. Since only one large pink colony from each cultivation method was included in the sequencing procedure the specific species presented for *Shewanella* cannot be regarded as representative for all colonies exerting the same morphology. However, it seems reasonable to conclude that all large pink and large white colonies belong to the *Shewanella* and *Pseudomonas* families, respectively. It should be noticed that one of the glass-like colonies originally picked from LH $CO_2$ after aerobic isolation turned pink and hence turned out to belong to the *Shewanella* family. This observation might influence the reliability of LH $CO_2$ as a selective medium for P. *phosphoreum*. Regarding the single intestine-like and the yellow colony found on LH Aer after prolonged storage at room temperature these were only present in one copy and could be identified as members of the *Psychrobacter* and *Arthrobacter* families, respectively.

**Table 6. Bacteria identities obtained from alignment of sequence data using BLAST and RDP Classifier. The number of identified sequences of each macroscopic morphology is displayed in brackets.**

| | Macroscopic morphology | Identity using BLAST and RDP Classifier |
|---|---|---|
| **LH 1% NaCl Aer** | Large pink colonies (1) | *Shewanella baltica* |
| | Large white colonies (1) | *Pseudomonas sp.* |
| | Small glass-like colonies (12) | *Photobacterium phosphoreum*<br>*Photobacterium iliopiscarium*<br>*Photobacterium kishitanii*<br>*Photobacterium aquimaris* |
| | Intestine-like colonies* (1) | *Psychrobacter cibarius*<br>*Psychrobacter uratirovans* |
| | Yellow colonies* (1) | *Arthrobacter psychrochitiniphilus* |
| **LH 1% NaCl $CO_2$** | Small glass-like colonies (34) | *Photobacterim phosphoreum*<br>*Photobacterium iliopiscarium*<br>*Photobacterium kishitanii*<br>*Photobacterium aquimaris* |
| | Large pink colonies** (1) | *Shewanella putrefaciens*<br>*Shewanella morhuae* |

*Present first at another inspection after prolonged storage at room temperature

**Originally glass-like but turned pink after isolated in aerobic conditions

All glass-like colonies from both cultivation techniques (except for the one that turned pink at isolation) were identified as members of the *Photobacterium* family. However, alignment using BLAST and RDP Classifier did not result in the identification of any specific *Photobacterium* spp., but instead four possible species were suggested (see Table 6). It should be mentioned that glass-like colonies, unlike the other three *Photobacterium* species, only were proposed as *P. aquimaris* in a few cases. In an attempt to distinguish the four species from each other a neighbor joining phylogenetic tree (including two copies of known 16s rRNA sequences of each specie available at the National Center for Biotechnology Information, NCBI) was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) v.6 software program (available free of charge at www.megasoftware.net). The resulting tree, with six sequences removed due to poor quality of sequence data (3 glass-like from LH $CO_2$, 2 glass-like from LH Aer and the large white colony from LH Aer identified as a member of the Pseudomonas family), is displayed in Figure 2.

As expected, glass-like colonies are branched together regardless of cultivation technique and are clearly separated from colonies with other macroscopic morphologies. However, one glass-like colony cultivated on LH $CO_2$ is undoubtedly clearly distinguished from the remaining ones (i.e. in the bottom of Figure 2). Since both good quality sequence data and reliable alignment results were observed for this colony, it is nevertheless hard to find a reasonable explanation for this observation. Regarding the four different species of *Photobacterium* it is obvious that they are genetically closely related and it is based on presented results therefore difficult to link the individual glass-like colonies to any specific specie. This observation is confirmed by Urbanczyk et

al. (2011) who managed to divide the closely related strains using multi-locus sequence analysis including genes coding for house-keeping proteins in addition to rRNA genes. According to the same reference *Photobacterium* species have multiple copies of the 16s rRNA gene and conserved parts of the gene evolve slowly, which potentially complicates the discrimination among recently diverged species based on single locus analysis.

Even though performed 16s rRNA sequencing turned out not to be enough to identify glass-like colonies as *P. phosphoreum* the fact that this specific specie today is generally accepted as the SSO of MA-packed cod (Dalgaard et al. 1993, Dalgaard 1995a, Macé et al. 2013 & Carrascosa et al. 2014) strongly strengthens the possibility that the glass-like colonies belong to this specie. Moreover, the fact that *P. iliopiscarium* is non luminescent and that *P. aquimaris* is normally found in warmer marine waters outside Japan (Urbanczyk et al. 2011) also points to this suspicion. Furthermore, Urbanczyk et al. stresses that *P. kishitanii* is known as a deep-sea fish symbiont whereas *P. phosphoreum* typically occurs in colder open ocean seawaters like the Atlantic Ocean where the fish used in this study was caught. Based on presented information and the fact that a more detailed identification procedure was not possible to perform within the limited timeframe of this thesis work, it was assumed that glass-like colonies present on LH plates are *P. phosphoreum* and hence will be regarded as so in following studies.

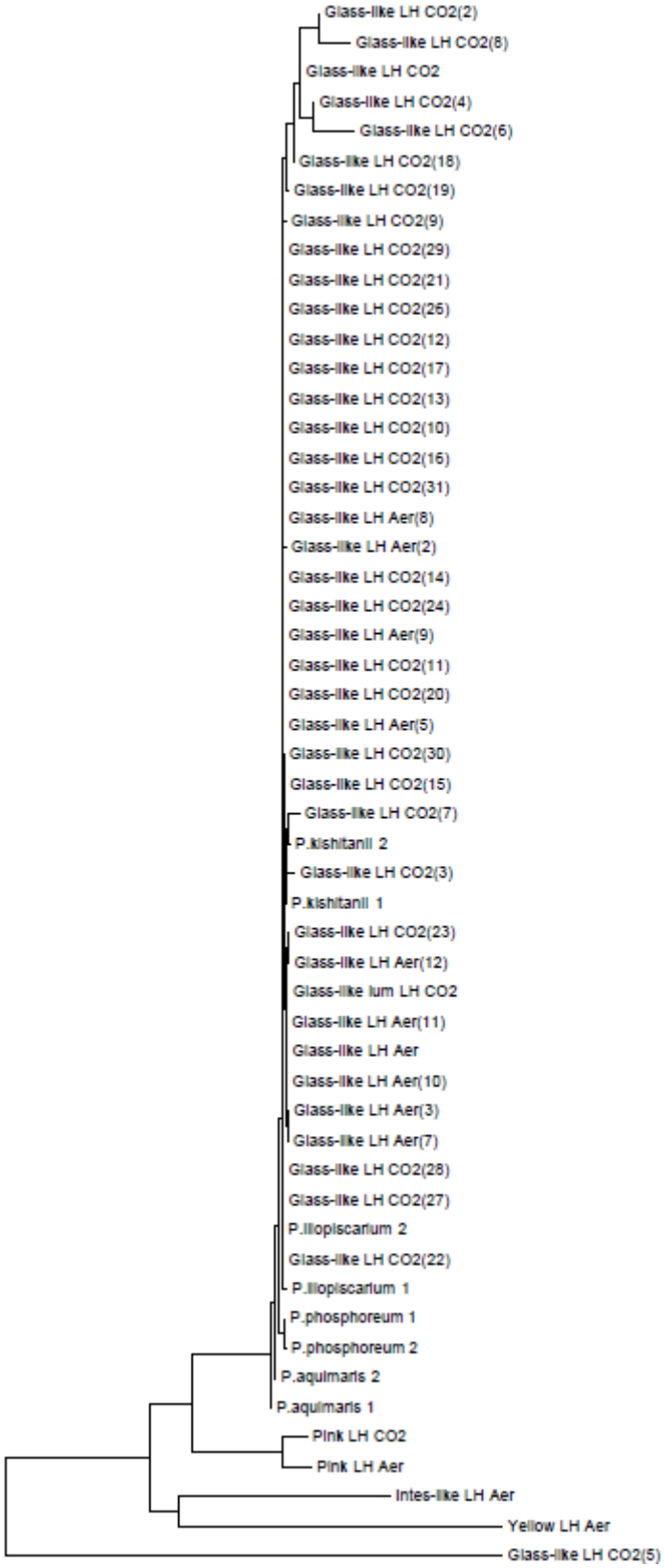


**Figure 2. A neighbor joining phylogenetic tree for colonies isolated from Long and Hammer´s medium incubated aerobically (LH Aer) and in 100% carbon dioxide (LH $CO_2$). Two copies of known 16s rRNA sequences for the four *Photobacterium* species suggested from BLAST and RDP Classifier searches are also included.**

## 4.2 Long term storage studies

### 4.2.1 Study number one

Based on results from initial studies LH 1% NaCl Aer and LH 1% NaCl $CO_2$ were applied in an attempt to construct growth curves for *P. phosphoreum* at two storage temperatures (2°C and 4°C). To be able to also study the development of $H_2S$ producing *Shewanella* sp. (which occur as black colonies on IA pour plates) IA 0.5% NaCl Aer was also included in the study.

Gas measurements of fish packages prior to microbial analysis revealed almost exclusively oxygen levels of <0.5%. Furthermore, a general trend of decreasing oxygen levels with storage time was observed, indicating bacterial oxygen consumption during storage. The level of carbon dioxide varied between approximately 25-40% depending on storage time. Gas measurements of bags containing LH 1% NaCl $CO_2$-plates performed at packaging revealed an actual carbon dioxide level of approximately 94-97% whereas a residual oxygen level of about 0.5-1% was observed. The levels of $CO_2$ remained almost intact during incubation (decreased <5%) and the oxygen content displayed a somewhat declining trend.

Results from microbial analysis are displayed as growth curves in Figure 3-5 below. The bacterial floras observed correspond to those found in initial studies, see Table 3 section 4.1.1. Surprisingly, no major differences were observed between 2°C and 4°C of storage and hence only growth curves for the latter storage temperature are included. Regarding the aerobic LH plates, glass-like colonies were (in accordance with initial studies) difficult to distinguish from other colonies during the first days of storage. The number of glass-like colonies for day 0 and day 1, presented in Figure 3, must therefore be considered as estimated values. As can be seen in the same figure both total growth and growth of glass-like colonies display the shape of a typical bacterial growth curve without any clear lag phase. Moreover, growth appear to be fairly similar for cod fillets A and C and glass-like colonies seem with few exceptions constitute an increasing proportion of the bacterial flora during time of storage.

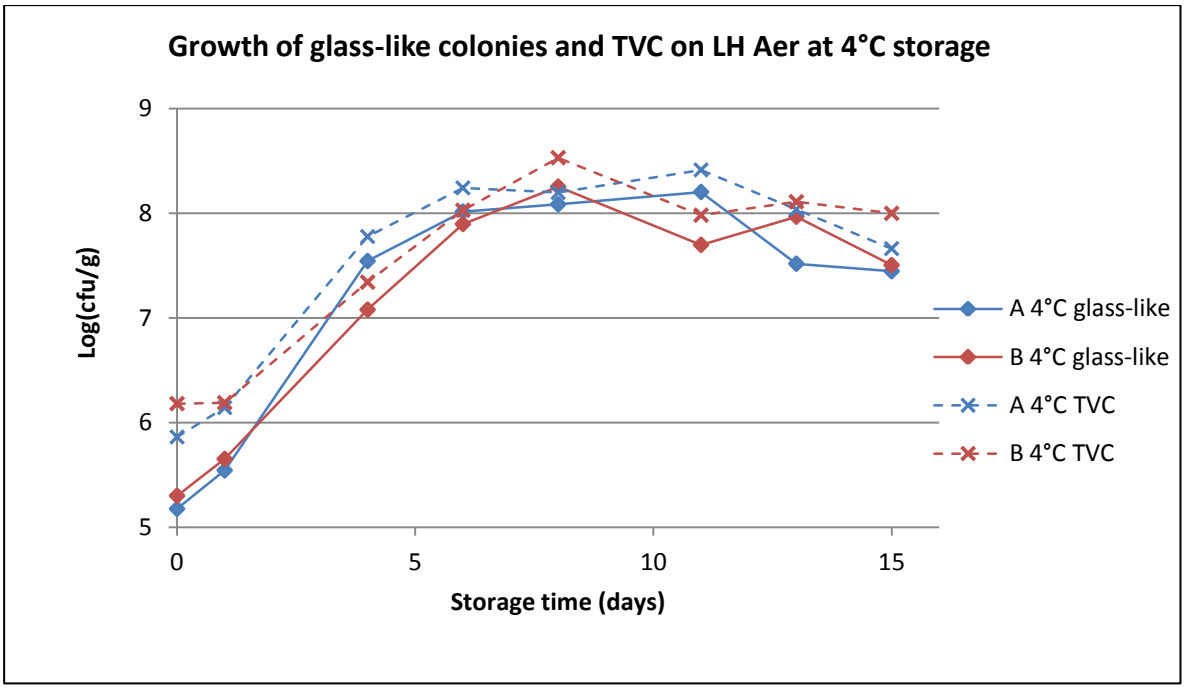


**Figure 3. Growth of glass-like colonies as well as total viable count (TVC) in two naturally contaminated cod fillets (A and B) stored in an atmosphere of 50% $CO_2$ and 50% $N_2$ at 4°C. Cultivation was performed on Long and Hammer plates (1% NaCl) incubated aerobically in 15°C for 6 days.**

The number of glass-like colonies found on LH Aer is for both fish fillets throughout the whole storage period in the same region as the total number of colonies observed on LH plates incubated in carbon dioxide (see Figure 4), indicating an equivalence between these cultivation techniques. However, LH Aer seem to display a higher bacterial number for day 0 and day 1 compared to LH $CO_2$, which points to the fact that the unsure values of the first mentioned technique probably are overestimated. On the other hand, a persistent minor trend of higher bacterial number can be observed for LH $CO_2$ starting from day 4, indicating the possibility of growth of other bacteria than *P. phosphoreum*. This suspicion was unfortunately confirmed, since another inspection of cultivated LH $CO_2$ plates after a couple of days in air (i.e. in a normal atmosphere) revealed that a significant proportion of the glass-like colonies had (in accordance with the observation for one colony during sequencing) changed in appearance and instead turned pink. In other words, it could be concluded that the utilized $CO_2$ incubation of plates is not enough to completely inhibit pink *Shewanella* sp. and hence this cultivation technique had to be ruled out from further studies. Due to the high levels of $CO_2$ utilized in the experiments (94-97%) the lack of inhibition was unexpected, but can possibly be explained by the fact that an atmosphere of 100% $CO_2$ never was reached.

Based on above findings growth curves obtained from LH $CO_2$ cannot be used to determine an accurate initial cod concentration of *P. phosphoreum.* Furthermore, the growth curves for glass-like colonies on LH-plates incubated aerobically consist of too few measurements during the exponential phase in order to be useful for this type of calculation (since values for day 0 and day 1 must be excluded due to above discussed uncertainty and the maximum value of bacteria seem to be reached already after six days of storage).

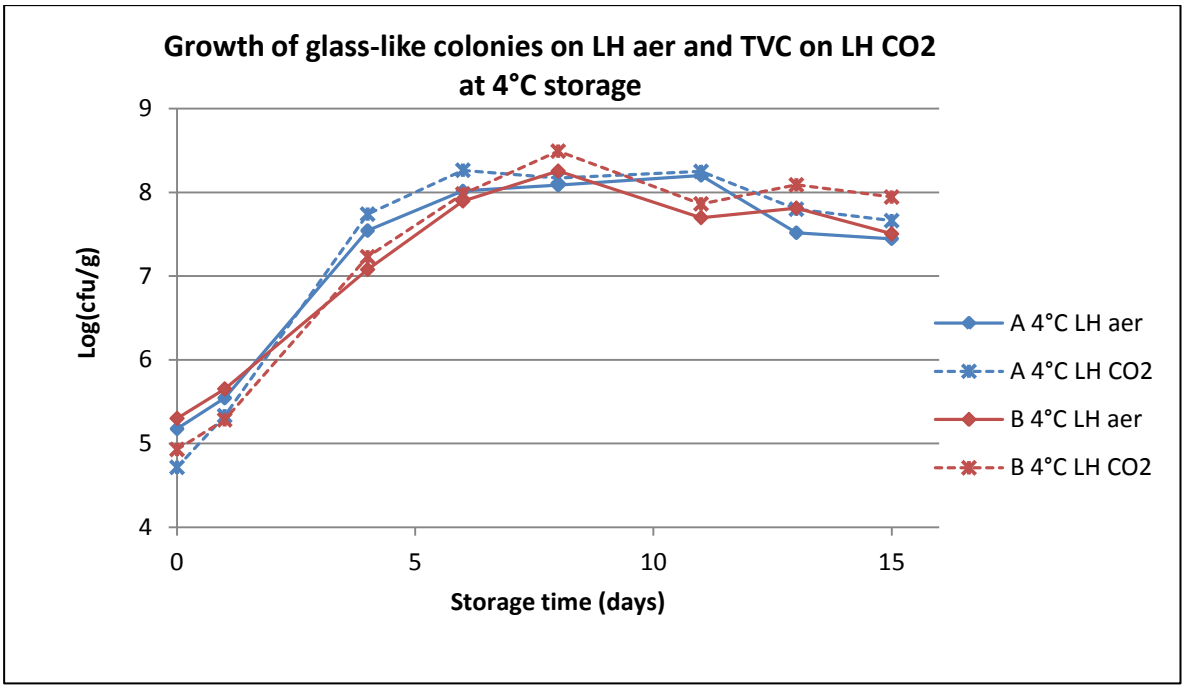


**Figure 4. Growth of glass-like colonies on Long and Hammer incubated aerobically compared to total viable count on the same medium incubated in 100% $CO_2$. Both cod fillet A and B were stored at 4°C in a modified atmosphere consisting of 50% $CO_2$ and 50% $N_2$.**

Regarding cultivation on IA pour plates a clear trend is the fact that the number of black *Shewanella* colonies increase in proportion during storage. As a result, almost exclusively black colonies exist already after 4 days, see Figure 5. Furthermore, fish fillet A seems to have somewhat higher bacterial numbers during the initial exponential growth face compared to fillet C. The maximum value however appears to be in the same region for both fillets and seems to be somewhat lower compared to the maximum numbers of glass-like colonies found for LH Aer in Figure 3.

Based on presented growth results it is obvious that although *P. phosphoreum* is characterized as the SSO of MA-packed cod $H_2S$-producing *Shewanella* sp. (identified as responsible for spoilage of aerobically stored cod) are also present in high numbers. However, sensory analysis did not reveal any smell of hydrogen sulphide during storage, indicating that the $CO_2$ present is enough to prevent these bacteria from causing spoilage of the product. On the other hand, a clear fishy character that with time increased in intensity could be observed starting from storage day 4, testifying that *P. phosphoreum* is the SSO of MAP cod. Regarding fish texture it went from tough and stringy during the first half of the storage period to more firm and dry towards the second half, due to release of water from day 8 and onwards. Moreover, the color of the fish pieces turned from white to more yellow/grey during the time of storage.

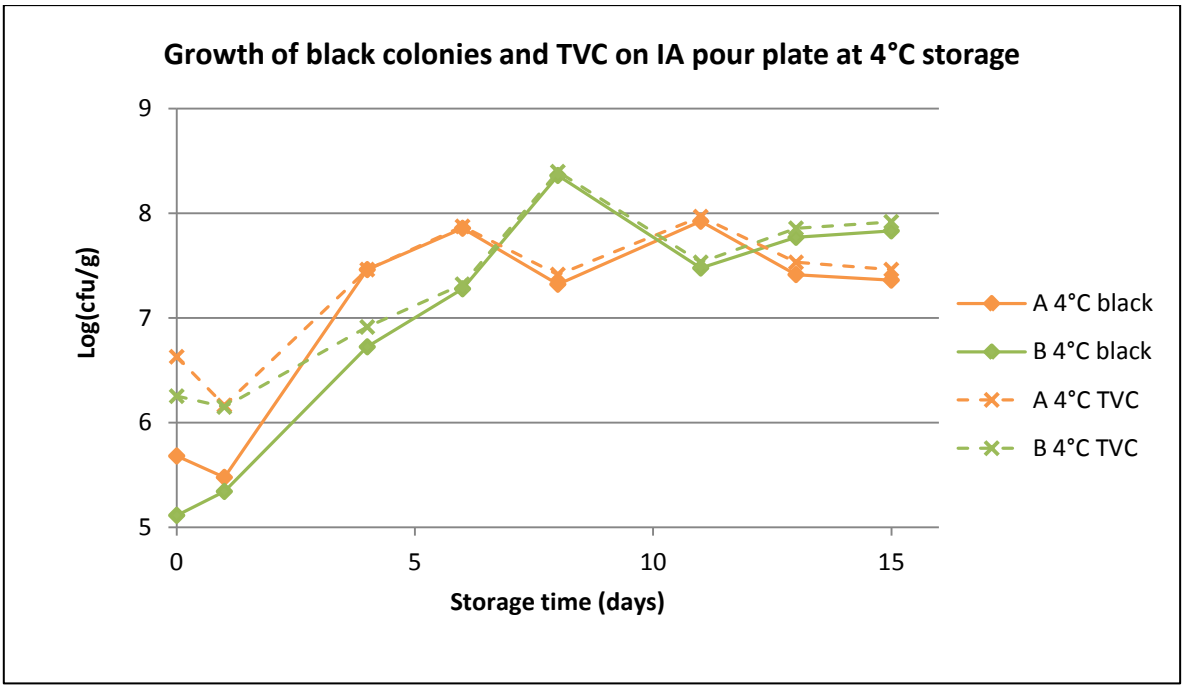


**Figure 5. Growth of black colonies and total viable count (TVC) in two individual cod fillets packed in 50% $CO_2$ and 50% $N_2$ and stored at 4°C. Cultivation was performed on Iron Agar pour plates (0.5% NaCl) incubated aerobically at 25°C for 3 days.**

### 4.2.2 Study number two

Due to lack of data during the exponential growth phase of *P. phosphoreum* observed in study number one, another storage study including measurements at day 0, 3, 4, 5, 6, 7 and 10 was performed. Since the cultivation technique involving LH plates incubated in carbon dioxide turned out to be less reliable (see section 4.2.1) and *Shewanella* spp. was excluded as spoilage bacteria of MAP cod in study number one, LH 1% NaCl Aer was the only technique included in this study.

Measurements of oxygen and carbon dioxide in fish packages prior to microbial analysis revealed levels in the same region as in the previous study ($O_2$<0.5% and $CO_2$ between 25-40%). Results of microbial analysis for glass-like colonies found in fillets A-E are displayed in Figure 6. In accordance with study number one glass-like colonies were difficult to distinguish from other colonies at day 0 and the reported concentration must therefore be considered as an estimate. As can be seen in Figure 6 the general impression is that the number of bacteria found in different pieces from the same fillet varies in the same magnitude as for fish pieces originated from different fillets (see the variance for one fillet within the stationary phase compared to the variance between individual fillets) and the maximum bacterial number are reached after approximately five days of storage.

Unfortunately, the constructed growth curves solely consist of data points representing the last part of the exponential phase (since values for day 0 must be excluded). In other words, linear regression cannot be used to derive a trustworthy initial concentration and data representing the initial part of the exponential phase need to be included in order to perform an accurate determination of the initial concentration of *P. phosphoreum*.

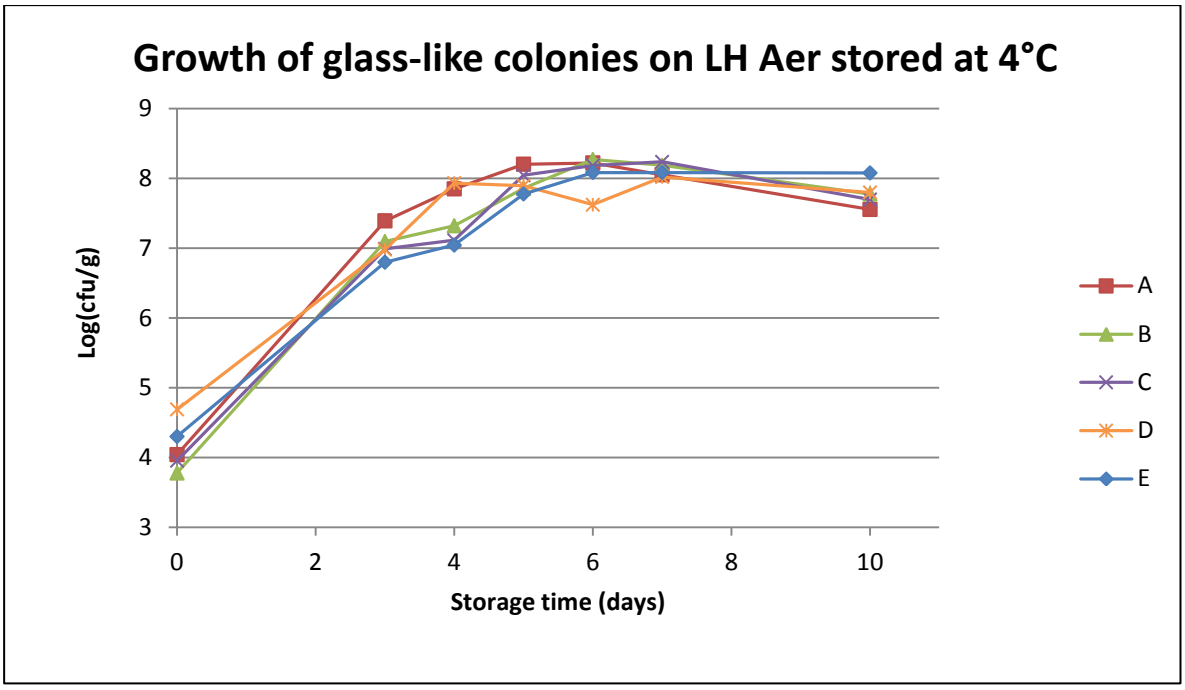


Figure 6. Growth of glass-like colonies in five individual cod fillets packed in 50% $CO_2$ and 50% stored at 4°C. The cultivation was performed using Long and Hammer´s medium incubated aerobically at 15°C for 6 days.

### 4.2.3 Study number three

In order to construct a *P. phosphoreum* growth curve that includes the total exponential growth phase another storage study was performed, utilizing the same cultivation technique as in previous study. Fish fillets were packed in modified atmosphere (50% $CO_2$/50% $N_2$) immediately after filleting at the fish distributor one day prior to deliverance and measurements were performed two times a day between day 1 and 7 and once at day 9. Based on the fact that similar results were observed for individual fish fillets in the earlier study biological replicates consisting of pieces from different fillets were utilized in this study.

Oxygen levels in fish packages prior to microbial analysis were in accordance with previous studies in most cases <0.5% whereas the level of carbon dioxide varied between approximately 30-45%. A possible explanation for the slightly higher $CO_2$-levels compared to earlier studies is the fact that a somewhat larger fish to gas ratio was utilized in this study (approximately 1:3 compared to 1:2).

Results from microbial analysis of the five biological replicates are presented in Figure 7. As in previous studies the difficulty in distinguishing glass-like colonies from other ones was observed at the first two sampling times (i.e. at day 1) and the reported values are therefore unsure. As can be seen in Figure 7, the deviation between replicates is small for all measurements and a maximum value just below 8 log (cfu/g) is reached after approximately 120 hours (i.e. 5 days).

By constructing an average growth curve based on the five biological replicates presented in Figure 7 the starting concentration of *P. phosphoreum* (at day 0) can be found by linear regression of the exponential growth phase (excluding the unsure values from day 1), see Figure 8. The calculated initial concentration of *P. phosphoreum* of approximately 2.15 log(cfu/g) is low compared to the values found from microbial analysis at day 0 in the two previous studies (about

4-5 log(cfu/g)). One must however keep in mind that the reported values for day 0 in previous studies are unsure, due to the difficulty in distinguish glass-like colonies from the remaining flora, and hence may be overestimated. Moreover, it is possible that lower starting values would have been observed in previous studies if it had been possible to utilize linear regression of the exponential growth phases. Nevertheless, the fact that earlier studies indicate higher starting concentrations means that more studies need to be performed in order to confirm the found starting concentration.

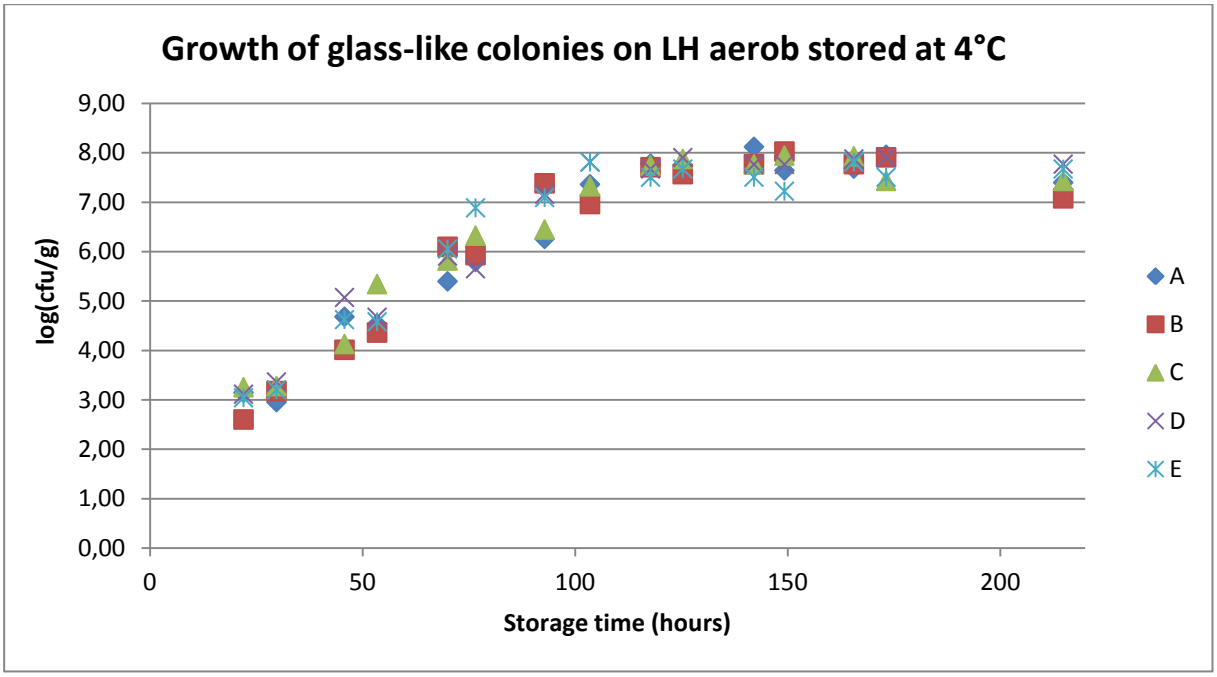


Figure 7. Growth of glass-like colonies on cod fillets packed in modified atmosphere (50% $CO_2$/50% $N_2$) immediately after filleting. Five biological replicates were included and the fillets were stored at 4°C.

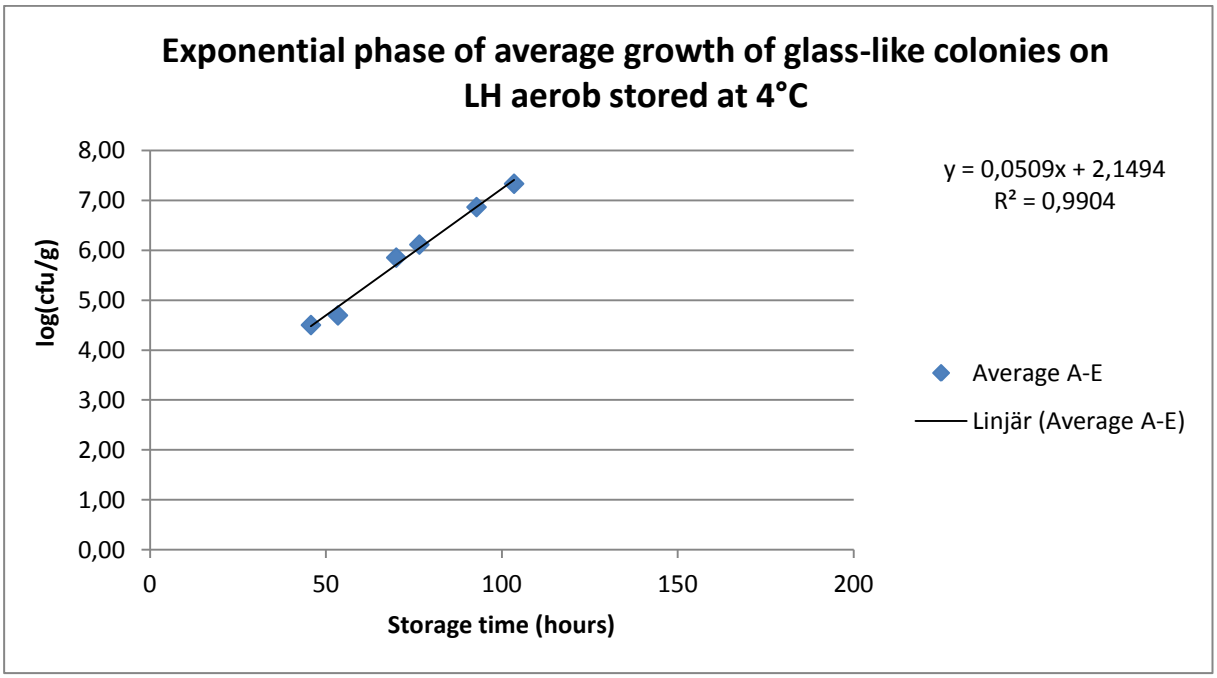


Figure 8. Linear regression of the exponential growth phase of average growth of glass-like colonies reveals a starting concentration of approximately 2.15 log (cfu/g) at day 0.

Even though the reported starting concentration of 2.15 log (cfu/g) seems reliable it is important to note that the value applies to the situation when the fish is MA-packed immediately after filleting. Moreover, it seems probable that the initial concentration will vary from batch to batch since the contamination of fish flesh depends on the specific filleting procedure, storage temperature, catch location etc. Furthermore, the fact that the initial modified atmosphere package was broken and fish fillets were repacked at arrival to SIK at day 1 is likely to exert a negligible effect on the presented results.

In order to validate the *P. phosphoreum* shelf-life model for MA-packed cod the initial amount of bacteria, storage temperature and percentage of $CO_2$ are used as input variables (see section 2.2.2). Regarding percentage of $CO_2$ it is important to use the concentration of carbon dioxide present in fish packages at equilibrium. According to Rotabakk et al. (2008) over 90% of the equilibrium concentration is dissolved in the fresh chilled fish flesh within 24 hours after packaging. Based on measured carbon dioxide levels 24-48 hours after packaging at Sik an average equilibrium carbon dioxide concentration of approximately 35% could be calculated for this study. With all input variables known the model can be used to predict growth of *P. phosphoreum* and hence construct a growth curve with data points corresponding to those found in the experimental study, see Figure 9.

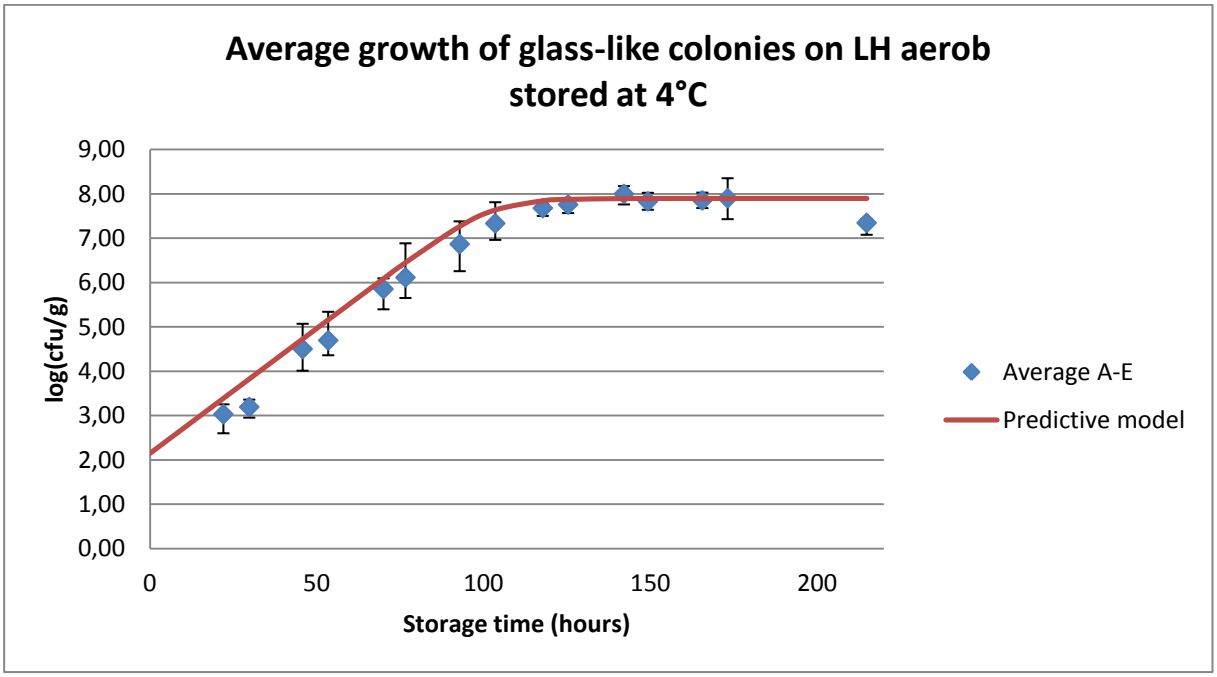


**Figure 9. Comparison of experimentally determined and model predictive growth of *P. phosphoreum* in cod packed in 50% $CO_2$ and 50% $N_2$ stored at 4°C. Error bars indicate highest and lowest bacteria concentration among five biological replicates.**

As can be seen in Figure 9, a good agreement is observed between experimental and predictive values. Nevertheless, the model seems to display somewhat higher bacteria concentrations during the exponential growth phase. A comparison of exponential growth reveals a specific growth rate of 0.0564 $h^{-1}$ for the predictive model compared to 0.0509 $h^{-1}$ for the experimental study. The difference in $\mu_{max}$ must be regarded as small, but a possible explanation for the observed deviation can be the fact that the utilized average $CO_2$ equilibrium concentration of 35% might not be representative for each individual fish package. Moreover, variation among

biological replicates results in presented average values, but as can be seen in Figure 9 almost all sampling points also include values that are higher than the once predicted by the model. Furthermore, it is important to keep in mind that we are dealing with biology and hence variations in bacterial growth between batches of cod caught at different times and places are not surprising due to variation in intrinsic factors (e.g. availability of nutrients).

As presented and discussed in section 4.1.3 performed partial 16s rRNA sequencing of bacteria growing on LH Aer and LH $CO_2$ was not enough to confirm that glass-like colonies exclusively are *P. phosphoreum*. The fact that the *P. phosphoreum* model accurately predicts growth of glass-like colonies (see Figure 9) however undoubtedly strengthens the assumption that observed glass-like colonies indeed are the same bacteria as Dalgaard et al. identified as the SSO of MA-packed cod. Consequently, it therefore seems reasonable to conclude that the model accurately is able to predict growth of *P. phosphoreum* in MAP cod. Another possible scenario can however be that observed glass-like colonies actually consist of a mixture of *Photobacterium* species that hence all contribute to fish spoilage. This idea is supported by the fact that species discrimination by gene sequencing was not available at the 1990s when Dalgaard et al. identified *P. phosphoreum* as the SSO of MA-packed cod. In any case, it is obvious that the presented model accurately predicts growth of glass-like colonies in this study, but specie identification by multilocus gene sequencing needs to be performed in order to bring clarity into the presented issue.

# 5. Conclusions

The task of determining starting concentration of the specific SSO of MA-packed cod, known as *P. phosphoreum*, turned out to hold a number of difficulties. Initial cultivation and storage studies revealed aerobically incubated Long and Hammer plates as the most appropriate method for cultivation of glass-like colonies. Partial 16s rRNA gene sequencing identified the glass-like colonies as members of the *Photobacterium* family, but was unfortunately not enough to distinguish *P. phosphoreum* from other closely related *Photobacterium* species.

Based on the assumption that observed glass-like colonies are *P. phosphoreum*, performed long term storage study resulted in an initial concentration of 2.15 log (cfu/g) for fish fillets packed in an modified atmosphere immediately after filleting. Shelf-life model validation revealed that the model is able to accurately illustrate growth of *P. phosphoreum* and based on existing sensory rejection evaluation hence predict shelf-life of MA-packed cod.

To be able to reliably conclude that glass-like colonies cultivated on Long and Hammer plates belong to the specie *P. phosphoreum* future studies utilizing multilocus gene analysis needs to be performed. If the outcome of these studies reveals a mixed *Photobacterium* flora the respective species must be inoculated into fresh cod and spoilage development must be studied in order to confirm *P. phosphoreum* as the SSO of MA-packed cod and exclude other species contribution to fish spoilage. Last but not least, the performed long term storage experimental setup needs to be repeated in order to find a truly representative initial concentration of the SSO that hence can be used to accurately predict shelf-life of MA-packed cod in the DYNAHMAT project.

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