Application Note

Bacterial Count in Aquaculture

Quantify bacteria in minutes instead of days

Introduction

Aquaculture, or “aqua farming”, is defined by the Food and Agriculture Organization (FAO) as the production of fish, crustaceans, mollusks, and marine plants. Reaching 114.5 million tons in 2018, aquaculture is a fast-growing food-producing industry that currently supplies more than 50 % of worldwide produced seafood [1]. Many countries invest substantial resources into aquaculture, including China as the world’s largest producer, India, Vietnam, Japan, South Korea, Canada and Norway. The most important fish species produced in fish farming are carp, tilapia, catfish and salmon. With a production value of 17.1 billion USD in 2018, the Atlantic salmon (Salmo salar) is one of the most valuable fish species in aquacultures [1].

In a fish aquaculture, farming systems are carefully managed to avoid rearing failure due stress, diseases by bacterial infections, or mass mortality, and to achieve optimum fish production. Therefore, testing the water quality and bacterial environment has a huge economic importance for all aquaculture farms.

To mate and die the salmons swim upstream

Compared to a typical freshwater or marine fish, the life cycle of salmons is very interesting: Salmon are anadromous, meaning they hatch in fresh water, migrate to the ocean and then return right back to the place they were born to reproduce. This means salmons are born and die in freshwater rivers, but remain in the sea until they become sexually mature. Therefore, a salmon aqua farm must consider different salt concentration to grow salmons under natural and controlled conditions.

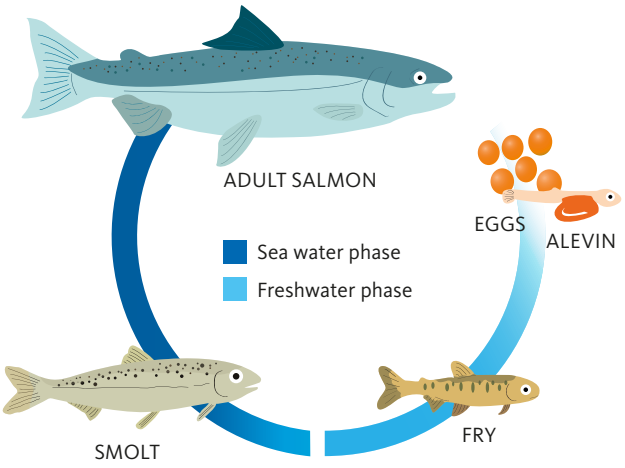

Fig. 1: Schematic illustration of the life circle of the Atlantic salmon.

The circle of life of a salmon in an aqua farm

Usually there are four phases in a salmon aqua farm: In phase 1 (salmon hatchery) the salmons start their lives in an incubator tray. Just like wild salmon, the fertilization of the roe takes place in freshwater. In phase 2 (first feeding) the young salmons "Fry" is being fed for the first time with pellets. In this phase the fry-salmons are transferred to a larger freshwater tank. In phase 3 (pre-smolt) the young salmons are facing a major change. A process known as smoltification leads to far-reaching physiological changes that enables the salmons to survive in salt water. After 10-16 months in freshwater, the salmon is ready to be placed into the sea at phase 4 (salmon on-growing). In this phase the salmon stay in floating sea cages or net pens until it reached its market-ready weight of 4-8 kg.

How to test against bacteria in sea water?

Bacterial pathogens in a salmon aquaculture are responsible for various diseases, including bacterial kidney disease (Renibacterium salmoninarum) [2] or piscirickettsiosis (Piscirickettsia salmonis) [3]. Therefore, testing the bacterial amount of sea water, intended as a guide for the optimal use of antimicrobial agents, is important for effective disease control at the different phases in a salmon aqua farm. Worldwide, the standard method for bacterial control in sea water is conventional plate count. However, this routine cultivation technique is limited by the delay of the results (1 – 14 days) and the statistically weak data interpretation (plate count only detects less than 0.1 % of the bacteria present in sea water).

Sysmex developed a new standardized, extreme fast, sensitive and semi-automated methods for the enumeration of bacteria in sea water: The CyStain™ BacCount reagents. This application note describes the advantages of this methods for a salmon aqua farm in Helgoland (Germany).

Fig. 2: Visual nature of the Atlantic salmon during different rearing phases.

Instrument requirements

The CyStain BacCount reagents use the CyFlow™ Cube 6 V2m Flow Cytometer (Ref. No. CY-S-3061R) – an easy-to-use and cost effective test system. The optional CyFlow™ Robby 6 Autoloading Station (Ref. No. CY-S-3083) allows a semi-automated enumeration of bacteria in water. The Robby is directly attached to the Cube 6 V2m Flow Cytometer and is able to pipette and to shake the samples using a 96 Well Plate.

Fig. 3: The CyFlow Cube 6 V2m Flow Cytometer with the CyFlow Robby 6 V2m Autoloading Station and the BacCount Viable kit.

Material and methods

Sysmex offers two different BacCount reagents: The CyStain BacCount Total kit enables detecting the total amount of bacteria – this is crucial to assess the microbial status of water. The CyStain BacCount Viable kit discriminates between live and dead bacteria, which is important to assess their physiological state. Both kits are designed for the quality control of water-based samples including sea water.

Kit components

CyStain BacCount Total kit:

- 5 Aliquots x 40 µL CyStain™ Green
- 29 mL CyStain™ Dilution Buffer

Fig. 4: CyStain BacCount Total kit (Ref. No. 05-5008).

CyStain BacCount Viable kit:

- 5 Aliquots x 40 µL CyStain Green
- 5 Aliquots x 400 µL CyStain™ Red
- 29 mL CyStain Dilution Buffer

Fig. 5: CyStain BacCount Viable kit (Ref. No. 05-5028).

Additional required equipment

- 96 Well Plate (Ref. No. 04-2020)
- 30 µm CellTrics filters™ (Ref. No. 04-0042-2316)
- Heating block/water bath set to 37 °C ± 0.5 °C
- Vortex mixer

Staining principle

The convenient staining principle allows a fast detection of bacteria in less than 15 minutes. The total cell count is based on the labelling of all bacteria with the membrane-permeant nucleic acid-binding dye CyStain Green. After excitation the fluorescence signal is counted for each individual microorganism. On the other hand BacCount Viable contains the additional CyStain Red dye. This dye is not membrane permeable and stains only bacteria with a damaged cell membrane. This is an indication of dead or dying cells.

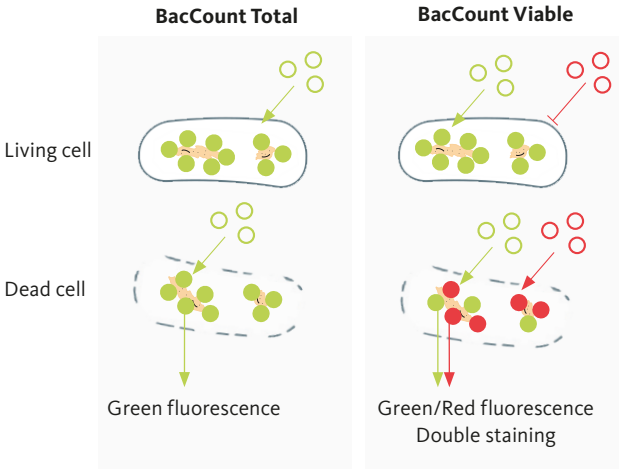

Fig. 6: Staining principle of BacCount Total (left) and BacCount Viable (right)

Sample preparation: Automated mode

NOTE: There are two options for sample preparation: Manual and automated sample analysis. In this Application Note all measurement were performed in the automated mode. For a detailed overview for manual measurements, please have a look at the Application Notes Total cell count (TCC) - [4] or Viable cell count (VCC) [5] of bacteria in water.

1a. Only for BacCount Total
Prepare a 10X working solution by diluting the 1000X stock solution of CyStain Green 1:100 with CyStain Dilution Buffer.

1b. Only for BacCount Viable
Prepare a working solution for both stains by diluting 20 µL stock solution of CyStain Green and 200 µL stock solution of CyStain Red with 1780 µL CyStain Dilution Buffer.

2. Mix 20 µL CyStain™ working solution with 180 µL water sample in a 96 Well Plate.

3. Mix gently by pipetting up and down the solution.

4. Incubate sample for 13 minutes at 37 °C ± 0.5 °C, protected from light.

Analysis range

The maximum cell number for an accurate data acquisition is 2 x 10^5 cells/mL. It is recommended to dilute a water sample if the cell number is higher. Dilute the water sample with ultrapure water or 0.1 µm filtered water before staining with the CyStain BacCount reagents.

Tab. 1: Analysis range of the CyStain BacCount reagents.

Detection Limit	Reportable Range
200 cells/mL*	1 x 10^3 – 2 x 10^5 cells/mL

*A specific treatment of the CyFlow Cube 6 V2m Flow Cytometer is required if the expected total cell count is lower than 1 x 10^4 cells/mL. Please refer to section General hints in the Application Notes Total cell count (TCC) - [4] or Viable cell count (VCC) [5] of bacteria in water for further information.

Data acquisition

NOTE: For a detailed data acquisition please refer to the Quality Check Manual [7].

1. Switch on the CyFlow Cube 6 V2m Flow Cytometer.
2. Enter your user name and passwort to start CyView™ software.
3. Load the script for automated analysis
4. Start the Priming procedure for an initial cleaning step of the device
5. Start the Quality Check (QC) procedure and follow the instructions on the display
6. Enter QC material information for LOT N° and expiry date on the automated FCS Express report
7. The analysis of stained water samples can be started after a “valid” QC procedure.
8. Insert a 96 Well Plate with your samples into the CyFlow Robby Autoloading Station and start measurement.
9. After the measurement FCS Express automatically starts and opens analysis report.
10. Start the Shutdown procedure after the sample analysis is completed and follow the instructions on the display.

Results

In cooperation with the Alfred Wegener Institute in Bremerhaven, water samples from tanks with different rearing phases of Salmo salar were analyzed by flow cytometry. According to the developmental stages of the fish, the water tanks contain ascending salt concentrations.

For analysis the water samples were stained with the CyStain BacCount Total or the CyStain BacCount Viable kit and measured automatically using the Cube 6 V2m Flow Cytometer and the Robby V2m Autoloading Station.

All water samples were passed through a 30 µm CellTrics filter to avoid clogging of the devices and diluted 1:100 with 0.1 µm filtered water prior to flow cytometric analysis to obtain results in the reportable range of this application.

Different disinfection methods were tested in the fish farm during the sampling period. For this purpose, the quality of the water was deliberately reduced. All sampled water tanks contained varying concentrations of viable bacteria whose influence is not emphasized here.

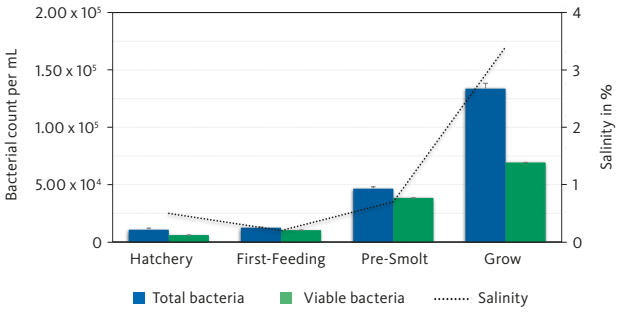

Fig. 7: Bacterial count of water samples during the different phases of a salmon rearing farm with appropriate salt concentration.

Conclusion

The CyStain BacCount kits are suitable for flow cytometric analysis of water samples with different salt concentrations without the necessity for special sample preparation. With the complete solution – devices and reagent kits – the quality of the water can be constantly monitored in aquacultures.

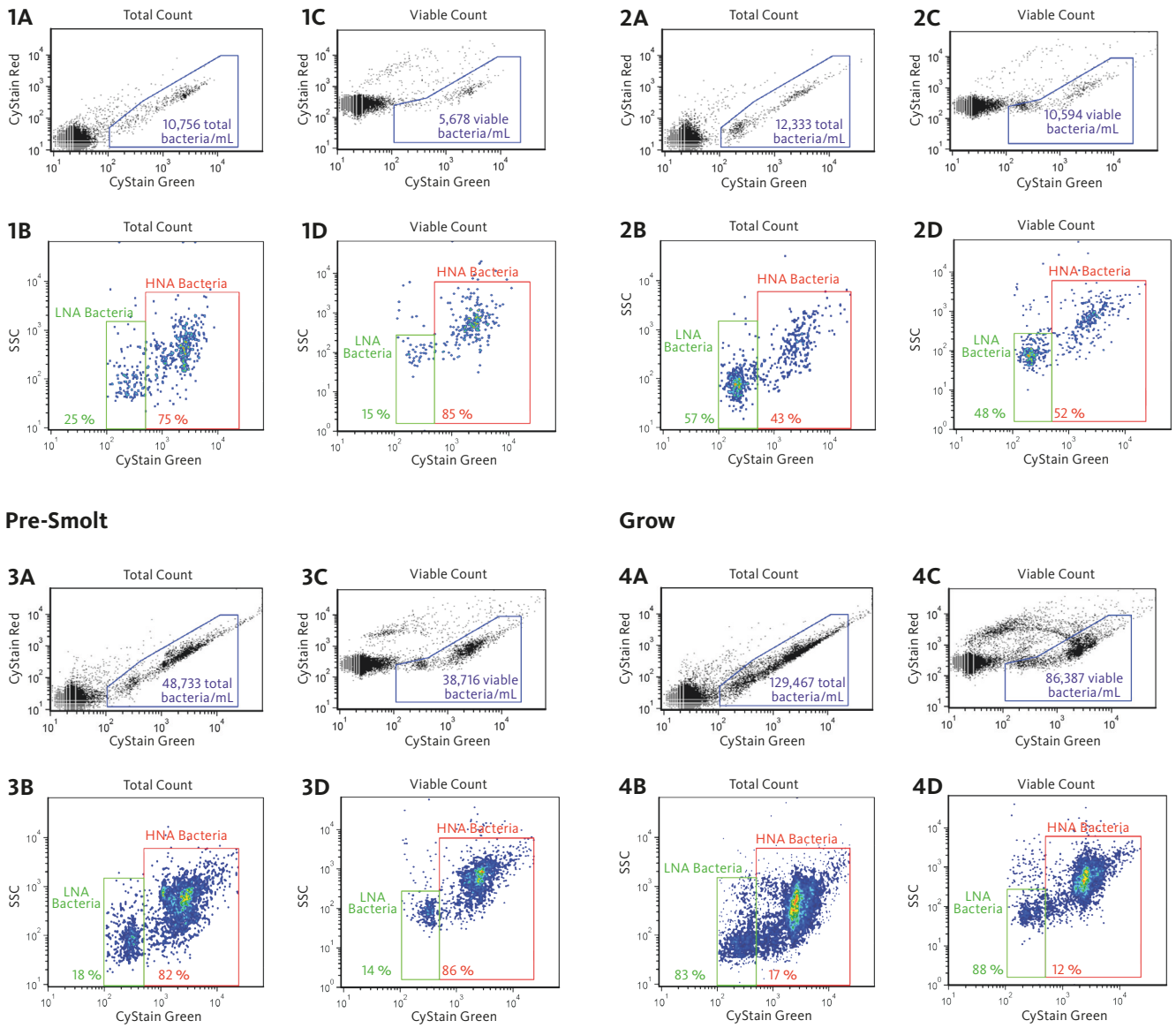

Fig. 8: Bacterial count of water samples during the different phases of a salmon rearing farm. All measurements were performed with CyStain BacCount Total (Total Count) or with CyStain BacCount Viable (Viable Count). (1) Salmon hatchery, (2) First feeding, (3) Pre-Smolt, (4) Salmon on-growing. (A) Dot plot showing CyStain Green vs. CyStain Red. All bacteria are located and automatically counted within the predefined blue gate. (B) Dot plot showing the side scatter (SSC) vs. CyStain Green of all bacteria. (C) Dot plot showing CyStain Green vs. CyStain Red. All viable bacteria are located and automatically counted within the predefined blue gate. (D) Dot plot showing the side scatter (SSC) vs. CyStain Green of all viable bacteria.

References

1. FAO, 2020. *The State of World Fisheries and Aquaculture 2020.*
2. Rhodes L. D., Mimeault C., 2019. *Characterization of Renibacterium salmoninarum and bacterial kidney disease*. Canadian Science Advisory Secretariat.
3. Rozas M., Enríquez R., 2014. *Piscirickettsiosis and Piscirickettsia salmonis in fish: a review.* J Fish Dis. 37: 163-88.
4. Sysmex Partec GmbH, 2020. *Total cell count (TCC) of bacteria in water.* Application Note.
5. Sysmex Partec GmbH, 2020. *Viable cell count (VCC) of bacteria in water.* Application Note.
6. Sysmex Partec GmbH, 2020. *CyStain™ BacCount reagents*. Quality Check Manual.

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