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Optimization of Sterilization Methods, Counting Rule and Yield Coefficient for AOC Determination Based on Flow Cytometry

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Abstract

Assimilable organic carbon (AOC) is an important and a well-accepted indictor for evaluating the biological stability in water. Flow cytometry combined with fluorescence staining now has become a most-used protocol. Sterilization methods and counting rules were applied by scholars are not uniform. Besides, the empirical value for yield coefficient is not rigorous. The paper mainly compared the disputes in AOC determination protocol. Firstly, sterilization method including membrane filtration, autoclaving, pasteurization, chlorination were compared. Pasteurization after 0.22μm membrane filtration could keep the integrity of bacterial cells and reveal the AOC value most accurately. Secondly, the counting rules of total cells counts and intact cells counts were compared. ICC avoids repeated counting of P17 during AOC measurement. Thirdly, control group with 0, 25, 50, 75, 100 and 200 μg Acetate carbon/L were conducted to confirm yield coefficient. The control group below 75 μg carbonic acid carbon/L were suggested.

Key words: AOC, sterilization method, counting rule, yield coefficient

1. Introduction

Assimilable organic carbon (AOC) is an important and a well-accepted indictor for evaluating the biological stability in water. During the last four decades, methods to determine AOC started from colony counting and gradually evolved (E. I. Prest, F. Hammes, M. van Loosdrecht, & J. S. Vrouwenvelder, 2016). The detection method proposed by Van der Kooij is the basis of the current AOC detection method, and researchers at home and abroad have improved and optimized it to varying degrees Flow cytometry (Wen, Wang, Huang, & Ma, 2014)combined with fluorescence staining now has become a most-used protocol, which make it possible to study biological stability accurately and quickly (F. A. Hammes & Egli, 2005).

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AOC detection generally comprises three processes: water sample pre-treatment, inoculation, and counting (Liu, Verberk, & Van Dijk, 2013). However, yield coefficient has always been confirmed through an empirical value 100 μg carbonic acid carbon/L provided by Van der Kooij, which is not always rigorous. At present, scholars still do not agree on the pre-treatment sterilization method and counting rules: (1) sterilization methods now used include membrane filtration, autoclaving, pasteurization, chlorination (Kinsey et al., 2017) ; (2) Two counting rules of total cell counts (TCC) and intact cell counts (ICC) could be found in references. These disputes have a great influence on AOC detection results.

Here we present an orthogonal experiment to explore the influence on AOC determination. The research focuses on that (1) confirm a reasonable range of values for control group to calculate yield coefficient. (2) study the differences among different

sterilization methods; (3) compare the counting rules of TCC and ICC.

2. Methods and materials

2.1 Preparation of carbon-free materials

Carbon-free glassware were prepared referring to the method of Hammes. The sample bottles and triangular flask were soaked in 0.2N HCl overnight, rinsed with tap water, distilled water and ultrapure water three times in sequence, air dried and then carbonized in a muffle furnace at 550 ℃ for 6 h. Other glassware was autoclaved at 121 ℃ for 20 minutes and air dried before use.

2.2 Yield coefficient determination

Pseudomonas fluorescens P17 and Spirillum NOX were utilised for testing bacterial in AOC determination. Carbon acetate solution (0, 25, 50, 75, 100, 125, 150 and 200 μg acetate C·L-1) were autoclaved and then incubated with P17 and NOX separately at the concertation of 10,000 cells/ml. P17 were counted after incubation for 3 days at 25℃ in dark while NOX were counted after 4 days.

2.3 Biological stability evaluation

Bacterial counts was determined with the support of flow cytometer (FCM, BD FACS Verse) by calculation of total cell counts (TCC) and intact cell counts (ICC) (Prest, Hammes, Kotzsch, van Loosdrecht, & Vrouwenvelder, 2016; Emmanuelle I Prest et al., 2016). The procedure consisted of two parts: staining and counting. Sample waters (500μL) were pre-heated to 35℃ for 5 min, then were stained with 5μL working solution containing SYBR® Green I (staining all cells) and propidium iodide (PI) (staining dead cells) (Peveler, Crisler, & Hickman, 2015). After incubation in dark for 15min at 35℃, stained samples were me assured on FCM (Li et al., 2018).

AOC was assayed with the method which was previously described by E. I. Prest, F. Hammes, M. C. van Loosdrecht, and J. S. Vrouwenvelder (2016). 40mL water samples were collected in 50mL carbonized bottles. Water samples contained residual chlorine were neutralized with thiosulphate solution and pasteurized at 70℃ for 0.5h. P17 was incubated into water samples at the concertation of 10,000 cells·mL-1 and was cultured at 25 ℃ in dark environment for 3 days to obtain ICC through FCM. Subsequently, P17 in water samples were killed with different sterilization methods. NOX were incubated in pasteurized samples and cultured for another 4 days. TCC and ICC of P17 and NOX measured were separately converted into AOC value as μg·L-1 acetate-Carbon.

2.4 Different sterilization methods for AOC determination

When AOC concertation is below 100 μg acetate C·L-1, the higher AOC detection accuracy is required (You, Lim, Hahn, Choi, & Gunasekaran, 2018). Pre-process operations in AOC detection including filtration and sterilization were studied through a pilot test. Indigenous bacteria were incubated into acetic acid carbon solution (sample A 50 μg·L-1, sample B 100 μg·L-1, sample C 150 μg·L-1) and cultured at the condition of 25 ℃ for five days. Then each sample was divided into seven groups to assay AOC values with different sterilization methods. Take sample A for example, A1 pasteurizing, A2 autoclaving, A3 chlorine sterilization, A4 filtration with 0.22 μm membrane, A5 filtration and pasteurizing, A6 filtration and autoclaving, A7 filtration and chlorine sterilization.

3. Results and discussions

3.1 Yield coefficient of P17 and NOX

The yield coefficients of P17 and NOX were confirmed as the follow formulas:

$$Y_{P17} = (G_{P17} - C'_{P17}) \frac{1000mL/1L}{100\mu g \ \text{Acetate}/L}$$

$$Y_{NOX} = (G_{NOX} - C'_{NOX}) \frac{1000mL/1L}{100\mu g \ \text{Acetate}/L}$$

in which, Y_{P17} is the yield coefficient of P17, G_{P17} is the P17 cells concertation of yield control group，C'_{P17} is the P17 cells concertation of blank control group，Y_{NOX} is the yield coefficient of NOX, G_{NOX} is the NOX cells concertation of yield control group, C'_{NOX} is the NOX cells concertation of blank control group.

Standard acetate carbon solution at the concertation of 75 and 100 μg acetate C·L-1 were separately utilized to calculate the yield coefficient of P17 and NOX.

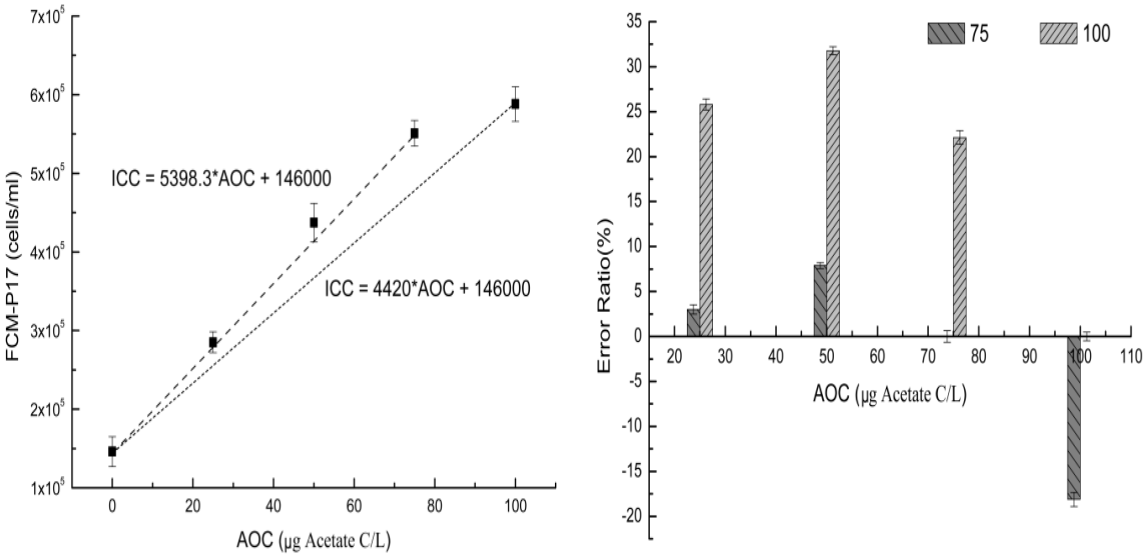

Fig. 1 Yield curves of P17 and error ration with different control group（75 and 100）

Then the yield coefficient was used to reverse the concentration of standard solution AOC by the yield coefficient. The measured values of AOC were compared with the real values. As shown in fig.2 and fig.3, the yield coefficient of P17 were 5.4×106cells/ml (set 75 μg acetate C·L-1 as the control group) and 4.4×106cells/ml (set 100 μg acetate C·L-1 as the control group). The error ratio of AOC calculated were lower in the former group where 75 μg acetate C·L-1 was set as the control group. The yield coefficient of NOX was 1.6×107cells/ml (set 75 μg acetate C·L-1 as the control group) and 1.2×107cells/ml (set 100 μg acetate C·L-1 as the control group). 80 μg acetate C·L-1 90 μg acetate C·L-1.

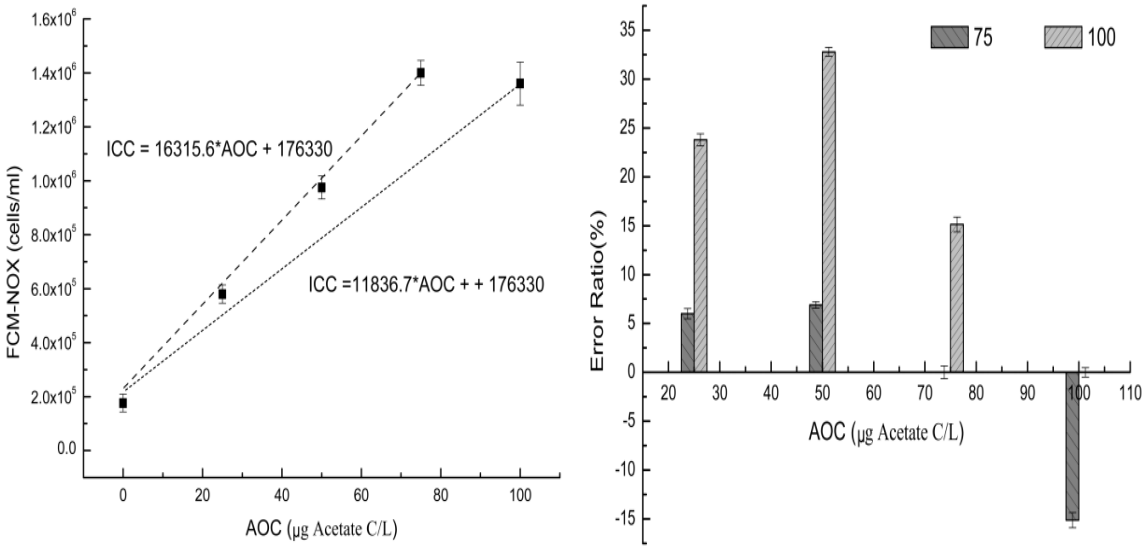

Fig. 2 Yield curves of NOX and error ration with different control group（75 and 100）

As shown in fig. 3, when the standard acetate carbon concentration is greater than 75 μg acetate C·L-1, the concentration of P17 bacteria decreased with the increase of the standard acetic acid carbon concentration, and the linear relationship with the standard acetic acid carbon concentration deteriorates. When the standard acetic acid carbon concentration is greater than 75 μg acetate C·L-1, the NOX bacterial concentration fluctuates within a certain range, and the linear relationship between it and the standard acetic acid carbon concentration also decreases. It was indicated that in the determination of low concentration AOC, it was not appropriate for the original method to directly use μg acetate C·L-1 sodium acetate standard solution for yield coefficient calculation

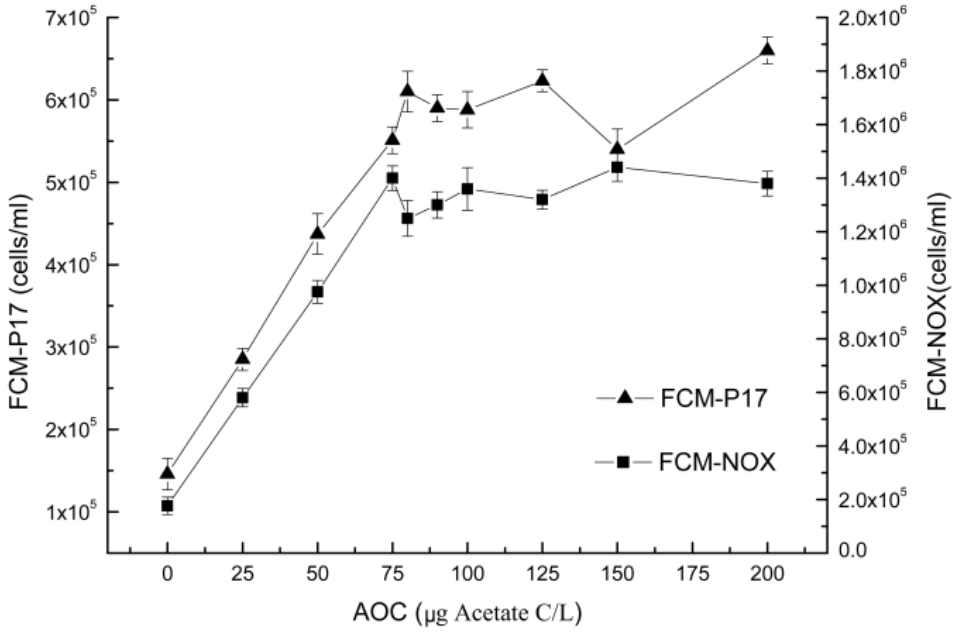

Fig. 3 Yield curves of P17 and NOX

After the growth plateau period, the AOC in the water is fully utilized by the bacteria, and the external nutrient concentration drops rapidly, and the water environment is not conducive to the continued growth of the bacteria. When intracellular catabolism significantly exceeds anabolism, bacteria begin to die in large numbers and enter a period of decline. Some bacteria may cause autolysis of the bacteria and release metabolites due to enhanced proteolytic activity; some Bacillus may release spores during this period. The higher the concentration of AOC, the faster the bacteria grow and the faster the plateau and decay. After entering the oscillating decay phase, the total number of bacteria assimilated by AOC in the 0-100 interval is weakly correlated with the linear relationship of AOC. When 100 μg Acetate C/L sample was taken out at the singular point, the linear relationship between 0-75 is good, so it could be speculated that under 75μg Acetate C·L-1, the carbon

limitation is obvious. When the AOC concertation is near 100μg Acetate C/L, the carbon may no longer be the only nutrient limiting factor, and nitrogen and phosphorus may also has a limiting effect on bacterial growth

3.2 Influence of TCC and ICC on bacterial counts

As shown in fig. 4, the correlation between the membrane treatment and sterilization methods and the TCC/ICC of the tested strains was significant, and the fluctuation of NOX was greater than that of P17. Under the conditions of successive inoculation of P17 and NOX, most of the AOC was used by P17. Only part of the aldehydes and carboxylic acids are used by NOX, and P17 is fully grown, but dead cells counts

(DCC) produced by membrane sterilization and DCC generated by competition (about 10%-20%).The NOX

in the next stage is included in its TCC. As shown in Fig. 5, the error is 16%-288% compared to the ICC count in the TCC count pair.

However, the error rate of the membrane is much higher than that of the membrane treated. The effect of pasteurization, higher sterilization and chlorine sterilization on the count of inactivated cells is more obvious in the case of the membrane, and the correlation between the membrane treatment and the sterilization method is significantly correlated with the TCC/ICC of the test species. NOX fluctuates more than P17. Under the conditions of successive inoculation of P17 and NOX, most of the AOC is used by P17. Only part of the aldehydes and carboxylic acids are used by NOX, and P17 is fully grown, but DCC produced by membrane sterilization and DCC generated by competition (about 10%-20%) The NOX

Fig. 5, the error is 16%-288% compared to the ICC count in the TCC count pair. However, the error rate of the membrane is much higher than that of the membrane treated. The effect of pasteurization, higher than sterilization, and chlorine sterilization on inactivated cell counts is more pronounced in the absence of membranes.

Therefore, the error rate of the ICC count is much smaller than the error rate of the TCC count. As shown in fig. 5, considering the AOC calculation results of the three groups A, B, and C, the water sample of the membrane was significantly higher than the membrane water sample, the chlorine sterilization result is high, the autoclaving is low, and the pasteurization is the largest. The degree of retention of cell integrity results in the closest result to its true value in the next stage is included in its TCC. As shown in

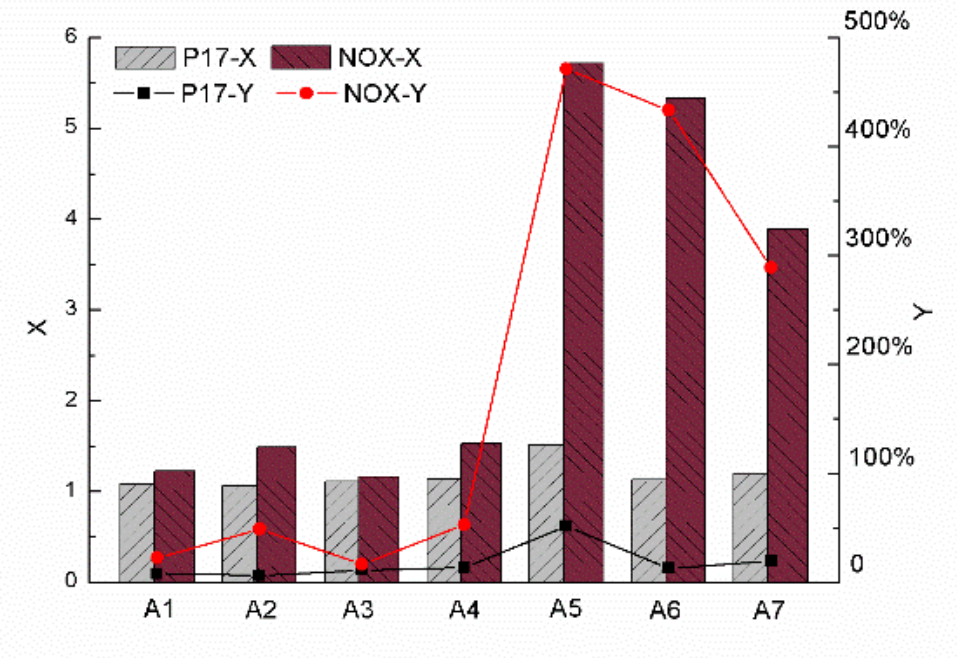

Fig. 5 Difference between TCC and ICC counts
$X = TCC / ICC, Y = (TCC - ICC) / ICC$

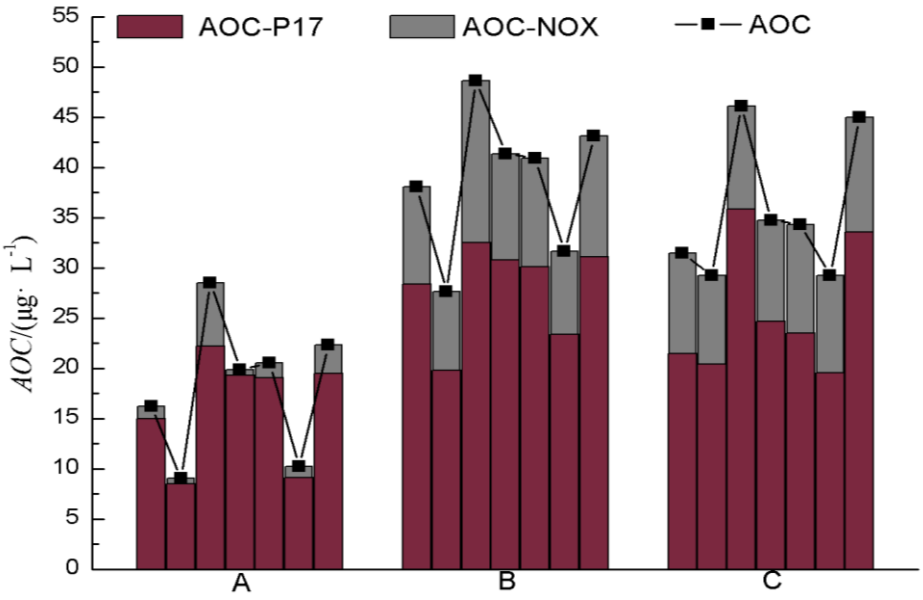

Fig. 4 AOC diversities among different groups

3.3 Effect of sterilization methods on AOC determination result

Fig. 6 revealed the differences among sterilization methods. It could be seen that the results of pasteurizing method were lower than that of autoclaving method, and lower than that of chlorine sterilization method. As shown in Fig. 7, there are two distinct clusters B1 and B2 in the A4 flow pattern. The flow patterns of the A1 and A4 curves show that the water sample intercepted by the filter membrane was inoculated after P17(F. Hammes, Berger, Köster, & Egli, 2010; van der Kooij, 1982). Live indigenous bacteria will have some repair and regeneration. P17 counts were obviously different between filtered samples and unfiltered samples. Filtration methods hardly had impacts on NOX counts.

It was certificated that pasteurizing with filtration method was the better choice to reveal the real situation in water. The possible reasons were that: (1) Part of dead cells were reused by P17 for regrowth while few was utilized by NOX. Yield values of NOX was much higher than that of P17 so NOX might have better adaptability to lower nutrition environment. Microbial competition was fiercer when nutrients were low and this would stimulate bacterial to utilize any possible substances including bacterium corpse. (2) Filtration methods could better preserve the qualities of DOC and remove all ICC, particularly the 0.22 μm filtration. Only few dead cells could go through the 0.22 μm membrane. (3) Nutrients were degraded more thoroughly during autoclaving process so that fewer substances were left for bacterial regrowth. Autoclaving achieved sterilization by changing cell membrane structure, inactivation of enzymes, denaturation of proteins, DNA damage through high-temperature steam. Autoclaving caused major inconsistent shifts in both qualitative and quantitative measurements of DOC, which led to the bacterial growth. (4) The effect of chlorine on microorganism lied in the lethal damage to nucleic acids. Hypochlorous acid is small could easily penetrate the cell wall, damage cell membranes, release proteins, RNA and DNA. As one kind of oxidants, chlorine could react with bacterial cells and dissolved organic matters to generate new AOC. A dramatic phenomenon in Fig. 7 was that the result of A7 is obviously higher than that in A3. The possible reason was that bacterial cells had higher priority than dissolved organic matters to react with disinfectant. Parts of DPBs in A7 sample were included in AOC after the reaction of chlorine.

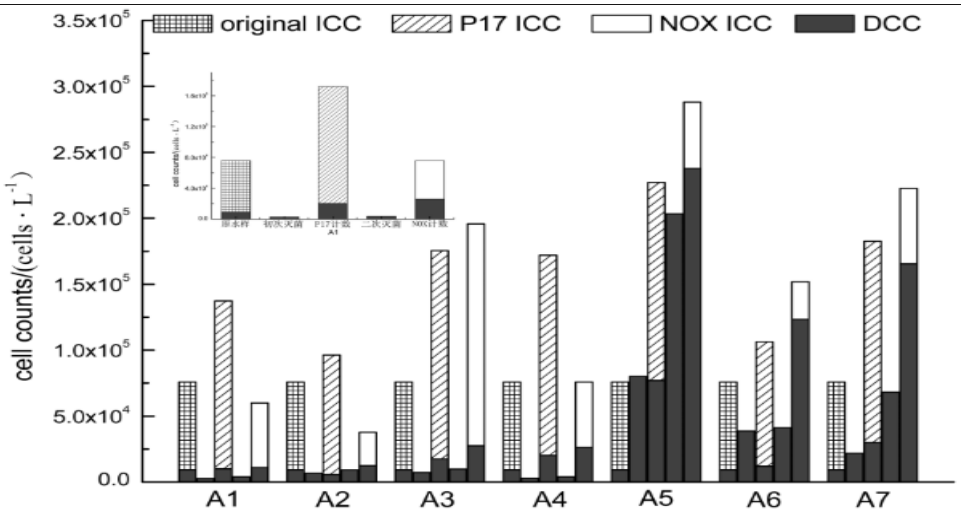

Fig.6 Diversities of TCC and ICC in water samples

The principles of pasteurization, autoclaving, and chlorine sterilizers vary, and the composition of organic carbon is affected by sterilization (Digitala Vetenskapliga Arkivet, 2017). Autoclaving was done by altering cell membrane structure, enzyme inactivation, protein denaturation, and destruction of DNA by high temperature steam. Under high temperature conditions, organic carbon undergoes incomplete degradation and complete degradation, resulting in low AOC detection results after autoclaving. The dyeing agent combines the low molecular organic matter produced after autoclaving to exhibit an impurity band in the shape of a rocket tail. The effect of chlorine on microorganisms was based on fatal damage to nucleic acids. Hypochlorous acid has a low molecular weight and can easily penetrate the cell wall, destroy cell membranes, and release proteins, RNA and DNA. As an oxidant, chlorine reacted with bacterial cells and dissolved organic matter to form small molecular weight AOC such as carboxylic acids, aldehydes, esters, and ketones, which makes AOC detection results high (Vázquez et al., 2011).

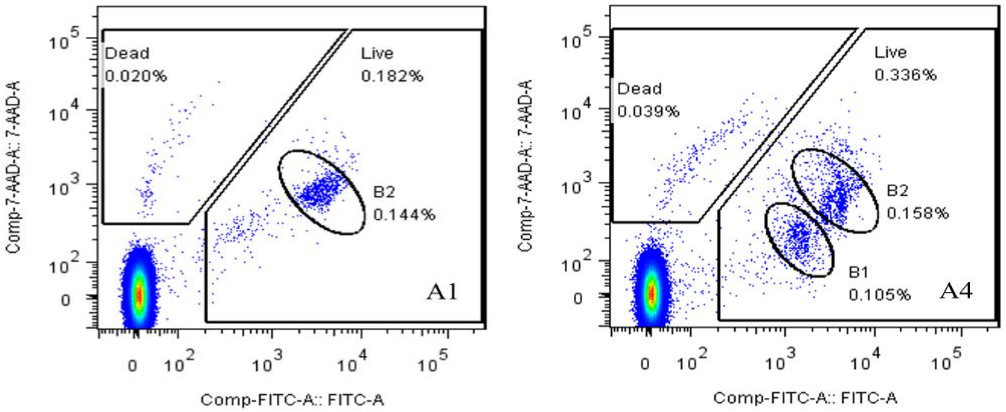

Fig. 7 Flow cytometry of P17 stained with PI and SYBR Green I

4. Conclusions

The AOC yield coefficient calculation using 75 µg Acetate C·L-1 would be more reasons in the low AOC value than that of 100 µg Acetate C·L-1.

The background bacteria in the water affect the accuracy of the determination of the biostability index, which can be eliminated by 0.22 µm filter memberance.

The method of autoclaving resulted a lower biostable value, while a high value resulted from chlorine sterilization method, and an optimal pasteurization method.

The AOC measurement results are more accurate using the value of ICC after membrane filteration, and TCC would repeatedly calculate the number of P17 inactivated cells.

5. Acknowledgements

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