Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2013.830683

52 (2014) 2734–2740
March

Taylor & Francis
Taylor & Francis Group

A review research of assimilable organic carbon bioassay

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Received 12 July 2012; Accepted 21 July 2013

ABSTRACT

Assimilable organic carbon (AOC) is one of the important parameters internationally recognized to assess the biostability of water. Studies at home and abroad on the AOC bioassay's development are reviewed from three aspects, including the selection of bacterial species for the bioassay, the optimization of inoculation method and incubation condition of water samples, and the evolution of bacterial growth measurement. The relationships between MAP (microbially available phosphorus), BRP (bacterial regrowth potential), and AOC are compared from determination mechanism. Moreover, the new development trend of AOC bioassay is predicted.

Keywords: Assimilable organic carbon; Bioassay; Biostability; Water quality

1. Introduction

Van der Kooij et al. [1] firstly proposed the concept of assimilable organic carbon (AOC) which was described as the organic compound limiting the growth of heterotrophic micro-organisms. The corresponding method for measuring the concentration of these compounds is known as AOC bioassay [1,2]. AOC [3,4] refers to the small portion of dissolved organic carbon (DOC) remaining in drinking water available as a source of carbon and energy for the growth of micro-organisms and reflects the microbial regrowth potential. AOC is known as an important indicator for measuring water biostability [5–7]. So far, the AOC bioassays have been applied in water treatment [8–10], water distribution system [11–13], reclaimed water treatment [14,15], and desalination of seawater [16,17], and have propelled the development of studies on water quality analysis.

Compared with the chemical method to measure total organic carbon (TOC) and dissolved organic carbon (DOC), AOC determination is a more complex biological method because AOC comprises a complex mixture of various compounds and is generally present in low concentrations in water. Van der Kooij [1,18] took *Pseudomonas fluorescens P17* (P17) and *Spirillum* sp. *NOX* (NOX) as test strains. Both P17 and NOX are common bacteria in water distribution system with the ability of using organic carbon compounds in low concentration in water. They require simple nitrogen source and no growth-stimulating substances, such as vitamins, and grow rapidly on solid media with distinguishable colony formation.

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Presented at the Second International Conference on Water Resources Management and Engineering (ICWRME 2012) Zhengzhou, China, 14–16 August 2012

Furthermore, they are easy to be maintained as pure cultures in the laboratory. P17 has great nutritional versatility in addition to carboxylic acids, while NOX can utilize groups of specific compounds, such as carboxylic acids. Due to their different nutritional capabilities, P17 and NOX are taken together as the test bacteria in AOC bioassay. AOC bioassay is based on the linear relationship between AOC concentration and bacterial maximum growth yield in water from inoculation to stationary phase. It uses maximum bacterial growth to calculate AOC value expressed in the equivalent form of acetate carbon (acetate-C) concentration. The maximum growth of test bacterium in tap water is converted to AOC value with a yield factor (Y) of the test bacterium on acetate-C, and the yield factor (Y) is derived from the slope of the standard linear curve. AOC bioassay is usually performed as an ''end-point'' measurement [19], and the water samples should be pasteurized before inoculation. Thus, bacterial batch culture and long-time process have to be applied to get the maximum growth of test bacterium. AOC bioassay is a time-consuming, complex, and laborious process [2,19,21,31]; therefore, it is an important problem how to shorten the determination time and simplify the procedures of AOC bioassay.

2. Development analysis of AOC bioassay

AOC bioassay was developed in 1982 [1]; thereafter, more and more studies contributed to optimize it constantly which mainly focused on three aspects: the selection of inoculum, the optimization of inoculation and incubation, and the evolution of bacterial growth measurements (Tables 1 and 2). In addition, microbially available phosphorus (MAP) and bacterial regrowth potential (BRP) were put forward as new biostability assessment parameters based on AOC bioassay.

2.1. Inoculum selection

Because the assay is based on the bacterial growth of pure cultures in a pasteurized water sample, selection and characterization of test bacterial strains is a major issue. The uses of a pure strain or defined mixed bacteria or even indigenous inoculum were selected in some studies (Table 1). The problem with one pure strain is that it cannot assimilate AOC completely in water due to lack of exoenzymes and interactions between different bacteria. Van der Kooij [1,18] added NOX to the single inoculum P17 to broaden the nutritional versatility instead of the pure strain P17. Kemmy [20] selected four specific strains including *P. fluorescens*, as inoculum to determine the AOC value in water samples; after five-day incubation, the bacterial growth became maximum which resulted in a shorter incubation period than the conventional AOC bioassay. Werner [35], Sathasivan [32], and Hammes [19] used indigenous micro-organism as inoculum. Although indigenous micro-organism as inoculum in AOC bioassay can utilize AOC more completely, for the complex exoenzymes necessary for utilizing high molecular weight organic compounds

Table 1
The international development of AOC bioassays [1,2,18–23,29–32,35]

Time (y)	Author	Inoculum	Bacterial growth measurement	Test time (d)
1982	Van der Kooij	P17	SPC	12–14
1986	Werner	IM	TM	2–4
1989	Kemmy	Four bacteria	SPC	5
1992	Van der Kooij	P17, NOX	SPC	12–14
1993	Kaplan	P17, NOX	SPC	5–9
1993	LeChevallier	P17, NOX	ATP-L	3
1999	Miettinen	P17, NOX	SPC	9
1999	Sathasivan	IM	TDC	5
2000	Escobar	P17, NOX	SPC	5–7
2004	Haddix	BM	BL	2–3
2005	Hammes	IM	TCC-FCM	5
2009	Weinrich	BM	BL	2–3
2011	Weinrich	*Vibrio harveyi*	BL	1–3

Symbols: y year, d day, BGM bacterial growth measurement, IM indigenous microorganism, BM bioluminescent mutants, SPC spread plate count, TM turbidity measurement, ATP-L adenosine tri-phosphate luminescence, L luminescence, BL bioluminescence, TCC-FCM total cell count with fluorescence staining and flow cytometry.

Table 2
The domestic development of AOC bioassays [24,25,27,28]

Time (y)	Author	Inoculum	Bacterial growth measurement	Test time (d)
2000	Liu, Wenjun	P17, NOX	SPC	6–7 or 9–12
2004	Shang, Junqiang	P17, NOX	SPC	6–7 or 9–12
2006	Fang, Hua	P17, NOX	SPC	6 or 9
2008	Bai, Xiaohui	P17, NOX	SPC	4–5

might not be present, and the complex interactions existing between the diversity of bacterial strains. As an indicator, the necessary characteristics are repeatability and comparability. Apparently, indigenous inoculum has some difficulties [15] into meeting the above essential requirements because of the undefined nature of the inoculum which makes quantification more complex. Recently, a new AOC strain (strain A3, Flavobacterium johnsoniae) was selected based on its presence in oligotrophic water environments and it utilized a group of compounds that includes oligo- and polysaccharides at microgram-per-litre levels in freshwater [36]; so, it further broadens the nutritional versatility of the defined mixed bacteria.

2.2. Optimization of inoculation and incubation

With regard to the optimization of inoculation and incubation of water sample, the studies mainly focused on the alternative of bacterial culture solution, the pretreatment of water sample, and the optimization of the conditions. In Kaplan's study [2] on AOC bioassay, it simplified the assay with a reduction in the incubation vessel size and the use of commercially precleaned glassware, and filtration of raw waters was recommended to remove most of the particular organic carbon prior to pasteurization. LeChevallier et al. [21] got a rapid method for the measurement of AOC by increasing the incubation temperature and increasing the inoculum density, which can quickly determine the bacterial growth potential of water within 2–4 days. In addition, Miettinen et al. [22] found that inorganic nutrients, particularly phosphorus, may limit the microbial growth in drinking water and the addition of an inorganic nutrient mixture (N, P, K, S, Ca, and Mg) to soft, humus-rich drinking waters could increase the AOC yield in most of the drinking waters which can remove the limitation effect of phosphorus. Escobar [23] optimized the storage conditions, and the study elaborated that samples heat treated at 72℃ for 30 min immediately followed by an ice bath for 30 min can be stored for at least Seven days without significant changes in AOC.

In domestic studies, Liu et al. [24] compared the effects on AOC bioassay of three inoculation methods which were independent inoculation [2] with each of NOX and P17 in each sample, simultaneous inoculation [18] with both of NOX and P17 each sample, and successive inoculation with firstly P17 for the P17 maximum growth determined, after being at 60℃ for 30 min, and secondly with NOX for the NOX maximum growth each sample and produced a new successive inoculatin different from the inoculation method produced by Paode and Amy [26], with firstly P17 for the P17 maximum growth determined, after being filtered, with secondly NOX for the NOX maximum growth in each sample (Fig. 1). Liu et al. [24] concluded that it is better to apply simultaneous inoculation and successive inoculation in AOC bioassay than independent inoculation (Table 3).

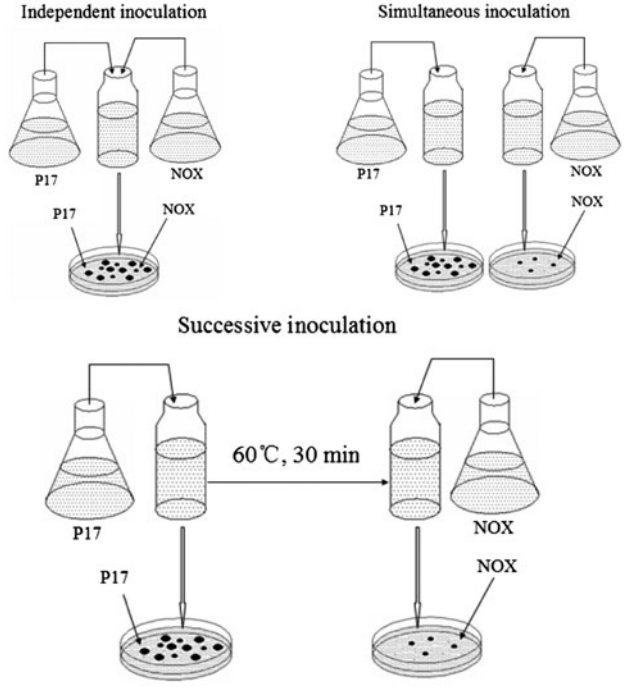

Fig. 1. Schematic drawing of three inoculation methods in AOC.

Table 3
The AOC values of water samples from three methods (cited from the paper of Liu [24])

Water sample		200 μg acetate-C L^{-1}	200 μg acetate-C L^{-1}	200 μg acetate-C L^{-1}	100 μg acetate-C L^{-1}	Water sample 1	Water sample 2
Independent inoculation	AOC_{P17}	200	200	200	100	235	207
	AOC_{NOX}	200	200	200	100	34	37
	AOC_{total}	400	400	400	200	269	244
Simultaneous inoculation	AOC_{P17}	223	234	228	120	238	202
	AOC_{NOX}	25	15	25	18	10	20
	AOC_{Total}	248	249	253	138	248	222
Successive inoculation	AOC_{P17}	200	200	200	100	235	207
	AOC_{NOX}	75	59	70	39	10	28
	AOC_{Total}	275	259	270	139	245	235

In Sang's et al. [25] study, the conventional procedure with successive inoculation produced by Liu et al. [24] used to measure AOC was modified to avoid the introduction of additional phosphorus into water sample by decreasing the amount of phosphate in the dilution buffer. Moreover, the AOC value measured by the modified procedure could indicate appropriately the bacterial regrowth potential in drinking water despite that either organics or phosphorus was the limiting nutrient for bacterial regrowth. Liu and Lu [33] introduced a Checklight AOC kit (Israel Checklight products) which can complete AOC bioassay in 2–3 h, but the test fee was higher than that of the conventional bioassay and no other relative reports had been found. Fang et al. [27] developed optimal AOC test conditions which can be used to detect AOC in a range of 10–300 μg acetate-C L^{-1} and put forth an improved method of calculating yield coefficient of organisms derived by different concentrations of acetate solution. Bai et al. [28] optimized the conditions of inoculation and incubation, and meanwhile modified the error resulted from successive inoculation which made the result more accurate by subtracting the increment due to lysis of the cells of P17 in the successive inoculation.

The results of these studies showed AOC parameter representing the bacterial regrowth potential determined by multiple factors. The flexible modification of AOC bioassay in application is helpful to judge the main limit factor of bacterial regrowth in a water sample by comparing the two AOC values determined with and without the addition of some kind of nutrient. Furthermore, the modification of AOC bioassay with or without the addition of some nutrient can provide information on the effect of the corresponding nutrient on the biostability of a water sample which leads to a better understanding of the relationship between water quality and biostability.

2.3. Evolution of bacterial growth measurement

At present, AOC bioassay quantifies bacterial batch growth by plate count, turbidity method, ATP luminescence, flow cytometry method, and bioluminescence as the number of colony-forming units (cfu) and cells, or other equivalent values in a water sample from inoculation until stationary phase. The main studies on the evolution of bacterial growth measurement are listed in Table 1.

The significant measurements of bacterial growth developed in the recent studies on AOC bioassay were ATP luminescence method, bioluminescence method, and total cell count with fluorescence staining and flow cytometry method. LeChevallier et al. [21] adopted ATP luminescence method into AOC bioassay, which reduced the test time to three days. Haddix et al. [29] used bioluminescent derivatives of P17 and NOX as inoculum in AOC bioassay, which can be an early physiological indicator of full cell yield due to peak luminescence, just prior to the onset of the stationary phase after exhaustion of AOC in a water sample. Moreover, Weinrich et al. [31] applied the bioluminescent derivatives of P17 and NOX as inoculum for AOC bioassay in reclaimed waters. According to Hammes' [19] study, total cell count with fluorescence staining and flow cytometry replaced plate count method can be used to establish complete growth curves for a natural microbial consortium growing on AOC. Weinrich et al. [30] used *Vibrio harveyi*, a marine organism that exhibits constitutive luminescence suitable for sea water and is nutritionally robust as AOC bioassay inoculum without construction of

bioluminescent AOC assay strains. Especially, the use of *V. harveyi* expanded the application field of AOC from freshwaters to seawater.

The bacterial growth measurement has developed from the plate count to the ATP luminescence and bioluminescence et al. in AOC bioassays. The development of the measurement made it more convenient to have a further understanding of bacterial growth dynamics. After the growth dynamics were studied by Van der Kooij [37] by measuring growth kinetics of pure cultures, later, Hammes [19] and Weinrich et al. [31] analyzed the bacterial growth dynamics in the AOC bioassays of the water samples by the measurements of fluorescence staining-flow cytometry and bioluminescence respectively. Hammes [19] concluded that growth measured only as cell numbers might err slightly in the estimation of total biomass increase, and Weinrich et al. [31] established a model to calculate bacterial maximum growth more quickly. However, ATP method reduces accuracy, and the flow cytometer counts cell numbers and not cell volume as the parameter. Since cells are different in size, the cell number is not appropriate to assess AOC quantitatively. Although the study on bacterial growth dynamics in AOC bioassay will attract more and more attention due to its ability of simulating bacterial batch growth in water, the development of bacterial growth measurement is still required.

2.4. Other parameters with same determination mechanism

For water biostability, there are many determinations. Except for AOC bioassay, Stanfield and Jago [38] measured ATP production of indigenous bacterial populations in water and Van der Kooij et al. [39] introduced a similar method for assessing growth potentials of piping materials or antiscalants for spiral-wound memberanes [40]. Among these parameters, bacterial regrowth potential (BRP) and microbially available phosphorus (MAP) are also based on the relationship between AOC concentration and the bacterial maximum growth from inoculation until stationary phase as AOC bioassay.

Sathasivan [32] proposed a new biostability parameter BRP and developed its measurement based on AOC bioassay in 1999. The main differences from AOC bioassay are two points: the indigenous inoculum instead of the inoculum of P17 and NOX and BRP value as the number of micro-organism cells of the maximum bacterial growth while AOC value as the equivalent acetate-C. In contrast, the indigenous inoculum has more ability to accommodate the native environment and assimilate AOC. Moreover, getting BPR value is more convenient than getting AOC value because it avoids establishing the standard curve between AOC and acetate and calculating the yield factor.

Lehtola et al. [34] also put forward another biostability parameter MAP for the phosphorus limited water. In MAP measurement, standardization was made with addition of different amounts of phosphorus (Na_2HPO_4) instead of acetate into the standard water with additional salt solution and 2,000 μg acetate-C L^{-1}. MAP measurement was based on the linear relationship between MAP value and the concentration of phosphorus (Na_2HPO_4); its yield factor also is the slope of the standard curve. It can provide more information of phosphorus in water sample with phosphorus as the standard substrate instead of acetate. However, based on the C: N: P molecular ratio of bacteria and biomass (100: 10: 1), the growth-determining factor in most waters is usually carbon, i.e. AOC [19]. These points emphasize the need for a reliable, realistic, and easily applicable AOC determination method.

2.5. Applications of AOC bioassay

Since AOC bioassays were produced by Van der Kooij [1], it has been applied widely in the studies on water treatment (including membrane treatment [40–43], advanced oxidation process (O_3,UV/H_2O_2) [9,44,45], and disinfection reagents [46,47]); water distribution system (including corrosion [13], pipe materials [48], and secondary contamination [11]); and reclaimed water treatment [14,15]; desalination of sea water [16,17] and biological risk [49–51] of water. It was in the studies on the regions where microbial growth in drinking waters was regulated by the content of phosphorus, rather than organic carbon [52–54] was developed. In the studies on reclaimed water and desalination of seawater, Weinrich et al. [31] built the AOC bioassay with the bioluminescent derivatives of P17 and NOX as inoculum for reclaimed water and another AOC bioassay [30] with *V. harveyi* suitable for sea water. Moreover, further developments [9,41,55] have demonstrated that at these low AOC concentrations the attached bacterial (biofilms) growth occurs at very short contact times. From the studies, it can be found that AOC bioassay develops with its application in the practical studies on water biostability in different situations.

3. Conclusions

Although MAP and BRP have strengthened the applicability of AOC bioassay, they also have limitations in some ways compared with AOC. AOC

is still the main parameter to evaluate water biostability. AOC bioassay is still time-consuming and complicated; in addition, there are some problems with interpretation and applicability of inoculum and yield factor. Therefore, future studies should be more on the collection of evidences so that improvements of the AOC methods add some essential knowledge to control regrowth problems. Moreover, improvements to AOC bioassay are required in future, in which the key aspects include evolution of bacterial growth measurement, inoculum selection, standard substrate option, as well as bacterial dynamics mechanism of AOC bioassay. Among the key aspects, evolution of bacterial growth measurement contributes enormously to better study of bacterial dynamics mechanism of AOC bioassay and is the basis for the study of inoculum selection and standard substrate option in AOC bioassay. Further simplifying the AOC bioassay mostly focuses on the study of bacterial growth dynamics in drinking water. Presently, with the help of advanced bacterial growth measurement, it will provide more and more information on bacterial growth dynamics in drinking water, and with the progress of research in this field AOC bioassay will be further optimized.

Acknowledgments

We are grateful to the National Natural Science Foundation of China for their financial support (No. 50908165).

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