Dialysis as a new pre-treatment technique for online bacterial counting

Short Communication

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Abstract

Real-time bacteriological counting technology is capable of providing an online profile of bacterial removal during the wastewater treatment process, and can enhance the safety of recycled water for potable water reuse. However, autofluorescence emanating from dissolved organic compounds present in treated wastewater interferes with the analysis. In this study, a novel approach is adopted, *viz.,* dialysis treatment for the removal of dissolved interfering substances from treated wastewater, and the efficiency of this treatment protocol is evaluated as a pre-treatment technique for real-time bacteriological counting. Dialysis using membranes having a molecular weight cut-off (MWCO) of 1000 kDa and 6–8 kDa were found to successfully reduce the intensity of autofluorescence emitted from the interfering substances; whereas the courser dialysis membrane having a MWCO of 1000 kDa was found to be more effective in removing the interfering substances. Here we demonstrate for the first time that continuous online dialysis treatment aids in the direct determination of the bacterial counts in ultrafiltration- and membrane bioreactor-treated wastewaters. The results of the study indicate that the dialysis pre-treatment technique is effective for continuously reducing the concentration of interfering substances in treated wastewater, and thus allows for direct online counting of bacteria.

Keywords: dialysis; wastewater; fluorescence spectra, bacterial counting; ultrafiltration.

1 Introduction

Microbial risk management of processed water is important to ensure the protection of public health in drinking water and in potable water reuse (Bailey et al., 2018; Barker et al., 2013). Microbial safety in drinking water and recycled water can be assured through periodical analysis of readily measured indicators (e.g., total or fecal coliform or *Escherichia coli*) (WHO, 2011; WHO, 2017). However, these conventional methods are time-consuming; thus, they are not capable of timely detecting the breakthrough of pathogenic microorganisms that can occur during the integrity breaches of water treatment processes. In contrast, online monitoring of bacterial concentrations in both feed and filtrate of a water treatment process can continuously provide a profile of bacterial removal, which enables to ensure its process integrity for bacterial removal (Asano and Cotruvo, 2004; CSWRCB, 2016). Speed, reliability, and frequency of analysis are the key for successful process integrity monitoring.

Online bacteriological counting techniques have attracted attention for process integrity monitoring purposes (Højris et al., 2016; Højris et al., 2018; Pepper and Snyder, 2016). Among the recent studies, flow cytometry, which determines bacterial counts through nucleic acid staining, has been increasingly assessed in water treatment. Flow cytometry is a technology which counts almost all the bacteria in water and can differentiate bacterial conditions (intact or damaged) by using multiple staining chemicals (Ou et al., 2017; Prest et al., 2014; Van Nevel et al., 2017; Whitton et al., 2018). Flow cytometry can also be mechanically integrated for online monitoring (Besmer et al., 2017). However, its requirement for continuous addition of expensive staining chemicals is a limitation for feasibility in full-scale operation. Another technology that has recently been applied to water treatment is real-time bacteriological counting. Briefly, it determines the bacterial counts without any chemical addition by detecting the intensity of (a) scattered light, which provides information about whether the particle size is greater than bacteria, and (b) autofluorescent light emitted from riboflavin and nicotinamideadeninedinucleotide hydrogen (NADH) in response to the excitation light (Ammor, 2007). However, the analysis using the fluorescence spectrometer is susceptible due to the presence of dissolved organics (e.g., humic acids or humic acid-like substances) in the surface waters and wastewaters. The autofluorescence emission from these substances can exceed the maximum detection limit of the fluorescence detectors and this hinders the counting (Fujioka et al., 2018).

Continuous pre-treatment of samples before real-time measurements remains a challenge. To date, only one technique, *viz.,* continuous dilution using pure water has been successfully demonstrated for effectively reducing the interfering substances (Fujioka et al., 2018; Fujioka et al., 2019b). However, the dilution method increases the limit of detection depending on the dilution rate. Here, we propose an alternative to overcome the aforesaid issues. We employed a dialysis pre-treatment technique, which is based on the passive diffusion of solutes from a high to a lower concentration through a dialysis membrane without a change in the solution volume. Constituents smaller than the membrane pore size, i.e. below the molecular weight cut-off (MWCO) of the membrane such as humic acid-like substances, are likely to pass through the membrane; whereas those larger than the pore size (e.g., bacteria) are retained in the sample stream, so that the treated sample may undergo bacterial counting without the influence of the background constituents. Though dialysis has been used to purify proteins and colloids, this approach has not been applied for the pre-treatment of real-time bacterial counting and its applicability remains unexplored.

This study is aimed to assess the efficiency of new pre-treatment technique, *viz.,* dialysis membrane treatment which is aimed at real-time counting of bacteria in treated wastewater. The assessment is conducted by (a) evaluating the reduction of interfering substances in treated wastewater using two dialysis membranes; (b) demonstrating the viability of online dialysis pre-treatment for continuous analysis of the two treated wastewaters.

2 Materials and Methods

2.1 Pre-treatment technique

The efficiency of reducing the background interfering substances in ultrafiltration (UF)-treated wastewater by dialysis treatment was evaluated by batch-scale experiments. The UF-treated wastewater was collected from a pilot-scale wastewater treatment plant, which filtered secondary wastewater effluent using an UF membrane module (SFP-2860XP, Dow Chemical, Midland, MI, USA). The secondary wastewater effluent was obtained from a primary settling tank and activated sludge process at a wastewater treatment plant in Nagasaki, Japan. The dialysis treatment system comprised of a SpectraFlo™ dynamic dialysis lab tank system with a capacity of 2200 mL (Repligen, Waltham, MA, USA), peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA), 20 L water reservoir for the dialysate, and a dialysis membrane with a flat width of 31 mm and length of 60 cm **(Fig. 1a)**. The two dialysis membranes used here were SpectraPor cellulose ester membrane (MWCO = 1000 kDa) and regenerated cellulose membrane (MWCO = 6–8 kDa) (Repligen, Waltham, MA, USA). Prior to the experiment, each membrane was soaked for 30 min in water and rinsed with pure water before use. A sample of 150 mL of UF-treated wastewater was filled in the dialysis membrane clamped with two dialysis tubing closures. The dialysis membrane was then submerged in the dialysis tank, and pure water was circulated at a flow rate of 0.5 L/min for 6 h. Thereafter, the treated sample in the membrane was collected for analysis.

[Fig. 1]

The effectiveness of dialysis treatment for continuous operation was evaluated online using a real-time bacteriological counter. The two batch of wastewater used here included the UF-treated wastewater and an effluent from a membrane bioreactor (MBR), which was collected at a wastewater treatment plant in Kitakyushu, Japan. A hollow fiber polyethersulphone dialysis membrane module (Diyalizerler Polynephron™ PES-25Dαeco, Nipro, Osaka, Japan) was used for the online test. The membrane module, which has an effective membrane area of 2.5 m^2, is designed for use in the renal replacement therapy of patients with kidney failure; hence, the membrane is capable of online operation. The continuous dialysis treatment system comprised of a peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA), smooth-flow pump (Q100, Tacmina, Osaka, Japan), 20 L water reservoir for dialysate, 200 mL glass bottle for treated wastewater samples **(Fig. 1b)**. Pure water prepared by filtering the tap water by a purification system (Mega Unity, Organo, Tokyo, Japan) was continuously fed to the dialysis module (i.e., outside the hollow fiber dialysis membrane) and recirculated at a flow rate of 0.5 L/min. Each test started by feeding the pure water to the feed side of the dialysis membrane module (i.e., inside the hollow fiber dialysis membrane), and the dialyzed-treated wastewater was transported to a real-time bacteriological counter. Thereafter, the pure water in the feed stream was replaced with the treated wastewater and the online counting continued for more than 10 min. Variance in bacterial counts through the dialysis pre-treatment was also evaluated by determining intact and damaged bacterial counts.

2.2 Analytical methods

The reduction in the interfering substances by pre-treatment method was evaluated using the excitation emission matrix (EEM) fluorescence spectra using RF-6000 spectrophotometer (Shimadzu Co., Kyoto, Japan). Online counting of bacteria was evaluated using a real-time bacteriological counter (IMD-W^{TM}, Azbil Corporation, Tokyo, Japan). This instrument measures bacterial counts based on the intensity of scattered and fluorescent light for the excitation (Ex) wavelength of 405 nm **(Fig. 1c)**. The intensity of auto-fluorescence emission (Em) is measured by two fluorescence detectors with EM wavelengths of approximately 415–450 and 490–530 nm. The maximum and minimum detection limits of the bacteriological counter at a sampling flow rate of 5 mL/min are 1 and 1.0×10^6 counts/mL, respectively. A previous study (Fujioka et al., 2019a) demonstrated a linear correlation of fluorescent particle counts in the range of 7.7×10^2–6.3×10^5 counts/mL between online bacteriological counter and epi-fluorescence microscopy. Intact and damaged bacterial counts of each sample were determined using a fluorescence microscope (BZ-X800, Keyence Co., Osaka, Japan). At first, 1 mL of the sample was stained with the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA), for 15 minutes in the dark at ambient temperature. The kit contains two dyes: SYTO®9—a green fluorescent nucleic acid that stains live and dead bacteria—and propidium iodide—a red fluorescent nucleic acid that stains only cells with damaged membrane. Thereafter, 200 µL of stained sample was filtered using a track-etched polycarbonate MF filter with 0.2 µm pore size (Merck, Tokyo, Japan). The filter was analyzed using a fluorescence microscope using a green filter (Ex wavelength = 470±40 nm, absorption wavelength = 525±50 nm) or a red filter (Ex wavelength = 545±25 nm, absorption wavelength = 605±70 nm). Intact bacterial counts were calculated by deducting the counts of damaged bacteria from total bacterial counts.

3 Results and Discussion

3.1 Removal of interfering substances

The effect of dialysis treatment on the removal of interfering substances was evaluated with EEM fluorescence spectra. According to a previous study (Fujioka et al., 2018), the major interfering substances for the real-time bacteriological countering are humic acid-like substances, which are detected at the Ex/Em wavelengths of 350/425 nm (Chen et al., 2003;

Liu et al., 2011; Nam and Amy, 2008). The Em light of humic acid-like substances can mask that of bacteria, which is detected using two fluorescent detectors at the Em wavelengths of approximately 415–450 and 490–530 nm for the Ex wavelength of 405 nm. In this study, the UF-treated wastewater effluent (referred to as no pre-treatment) showed noticeable fluorescence intensity at the Ex/Em wavelength of the fluorescent detectors **(Fig. 2a)**. Significant reduction in fluorescence intensity at the regions of the fluorescent detectors was observed for a 50-fold dilution **(Fig. 2b)**. Dialysis pre-treatment with a courser membrane (MWCO = 1000 kDa) led to a considerable reduction in fluorescence intensity at the detector's regions **(Fig. 2c)**. The dialysis membrane is expected to retain bacteria in the sample but allows the discharging of the interfering substances, because the pore size of the membrane with a MWCO of 1000 kDa is expected to be < 0.2 μm (Sarbolouki, 1982), which can reject small bacteria that can have a diameter of down to 0.2 μm (Gao et al., 2018; Heulin et al., 2003; Sahin et al., 2011). Another membrane with a smaller MWCO of 6–8 kDa led to a less reduction in fluorescence intensity than the courser membrane **(Fig. 2d)**, indicating that only a few interfering substances were removed through the tighter dialysis membrane due to its smaller pore size (i.e., more restricted passage) of the dialysis membrane. It is noted that the MWCO of these dialysis membranes is the original value unaffected by the treated wastewater matrix. The MWCO can vary according to the formation of a cake layer on the dialysis membrane surface or the clogging of the membrane pore, which typically occur due to impurities in a given water type, including treated wastewater during a long-term pre-treatment. As the reduced MWCO is likely to inhibit the transport of interfering substances, future studies should attempt to understand the changes in MWCO during a long-term pre-treatment.

[Fig. 2]

The reductions in fluorescence intensity by dialysis and dilution methods spanned over the Em wavelengths of 415–600 nm for a specific Ex wavelength of 400 nm **(Fig. 3)**. The results here indicate that commercial dialysis membranes can reduce the concentrations of the interfering substances (i.e., humic acids or humic acid-like substances) in a similar way to the dilution method. The reductions with the broad Em wavelengths also indicate that the pre-treatment method is likely to function with real-time bacteriological counters provided by other manufacturers, because riboflavin in bacteria, which is a key substance that allows for real-time bacteriological counting without stain addition, emits fluorescence at the Em wavelengths of approximately 475–575 nm (Naramura et al., 2013).

[Fig. 3]

3.2 Online analysis

A successful pre-treatment technique is expected to achieve a sufficient reduction in the concentration of interfering substances in wastewater by attaining a level that allows for online bacterial counting using the real-time bacteriological counter. Therefore, the effectiveness of the dialysis pre-treatment on mitigating the inhibition for online monitoring bacterial counts was evaluated using UF- and MBR-treated wastewaters. It is noted that the analysis of non-pretreated samples triggered an alarm of analytical failure because the intensity of the sample's autofluorescence exceeded the maximum capacity of the fluorescence detectors and immediately halted the analysis (the display image is not shown). Therefore, no analytical results were obtained for the analysis of the non-pretreated samples. UF-treated and MBR-treated wastewater after the dialysis pre-treatment showed 6.5–6.6 × 10^4 and 0.9–1.0 × 10^4 counts/mL, respectively **(Fig. 4a)**. The results indicate that the dialysis pre-treatment allows monitoring the bacterial counts of actual treated wastewaters online. Further, the variation in bacterial counts before and after the dialysis treatment was also assessed by examining changes in, intact and damaged bacterial counts using fluorescence microscopy. As a result, intact bacterial counts in the UF-treated wastewater before and after dialysis treatment were almost constant at 24–27 × 10^4 counts/mL, whereas those in the MBR-treated wastewater before and after dialysis treatment varied slightly in the range of 10–11 × 10^4 counts/mL **(Fig. 4b)**. The results indicate that the dialysis pre-treatment technique is capable of removing the dissolved interfering substance without major changes in bacterial counts, showing its viability as a pre-treatment of real-time bacteriological counter.

[Fig. 4]

It was observed that the bacterial counts determined by the real-time bacteriological counter differed from the intact bacterial counts determined by epifluorescence microscopy because their detection mechanisms are different. Intact bacterial counts determined by nucleic acid staining and epifluorescence microscopy fundamentally cover all of the intact bacteria regardless of their dimension. The real-time bacteriological counter is designed to count bacteria with a size larger than 0.3 μm and a certain intensity of autofluorescence emitted from riboflavin and NADH. Therefore, the real-time bacteriological counter is unlikely to count small (i.e., < 0.3 μm) or less active bacterial cells with low autofluorescence. As a result, the exclusion of these small or low-autofluorescence-intensity bacteria can cause the underestimation of online bacterial counts, as demonstrated in **Fig. 4**. Despite the difference, the data obtained here demonstrated that the dialysis treatment does not change bacterial counts but can remove the interfering substances in treated wastewater, allowing for the real-time counting of bacteria.

3.3 Technology implications

The results attained in this study showed that MWCO of dialysis membrane can be an important factor for the viability of the dialysis pre-treatment prior to online bacteriological counters.

Since, the molecular weight of organic substances in treated wastewater can be up to 400 kDa (Shon et al., 2004; Worms et al., 2010), the course dialysis membrane with a MWCO of 1000 kDa can theoretically remove almost all organics including humic acid-like substances in water. Dialysis membranes designed for batch-scale tests typically have a wide range of MWCOs in a manufacturer's line up; thus, the selection and optimization of a membrane's MWCO that is sufficiently large for the dialysis pre-treatment can be readily conducted. However, these batch-type membranes are not designed for online treatment. Almost all commercial dialysis membrane modules that can be operated online have been designated for the medical field (e.g., dialysis treatment for patients with kidney disease), and their details (e.g., MWCO) are not provided by the manufacturers. Understanding their detailed properties has the potential to facilitate the selection of commercial dialysis membranes suitable for water treatment applications. In addition, to verify the applicability of membrane's MWCO for the pre-treatment, long-term validations using different wastewater sources and dialysis membranes are to be carried out.

4 Conclusions

This study utilized the principle of dialysis to remove organic substances from wastewater that hinders the analysis during bacterial counting. Dialysis using membranes having a molecular weight cut-off (MWCO) of 1000 kDa and 6–8 kDa successfully reduced the intensity of autofluorescence emitted from the interfering substances in ultrafiltration-treated wastewater. It was demonstrated for the first time that continuous online dialysis treatment aids in the direct determination of bacterial counts in ultrafiltration- and membrane bioreactor-treated wastewaters without any dilution. Therefore, this study suggests that the dialysis pre-treatment technique is a viable option as a pre-treatment of real-time bacteriological counter.

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FIGURES

Fig. 1 – Schematic diagram of (a) batch-scale and (b) online dialysis treatment, and (c) the illustration of bacterial detection with scattered light (SL) and fluorescent light (FL) and the reduction of background interference substances.

Fig. 2 – Excitation emission matrix (EEM) fluorescence spectra of the ultrafiltration (UF)-treated wastewater: (a) no pre-treatment, (b) after 50-fold dilution, (c) after dialysis with 1000 kDa membrane, and (d) after dialysis with 6–8 kDa membrane.

Fig. 3 – Emission (Em) fluorescence spectrum at the excitation (Ex) wavelength of 400 nm.

Fig. 4 – (a) Online analysis and (b) intact bacterial counts before and after the dialysis pre-treatment of the ultra-filtration (UF)-treated, and membrane bioreactor (MBR)-treated wastewaters. Error bars represent the range of duplicate samples.

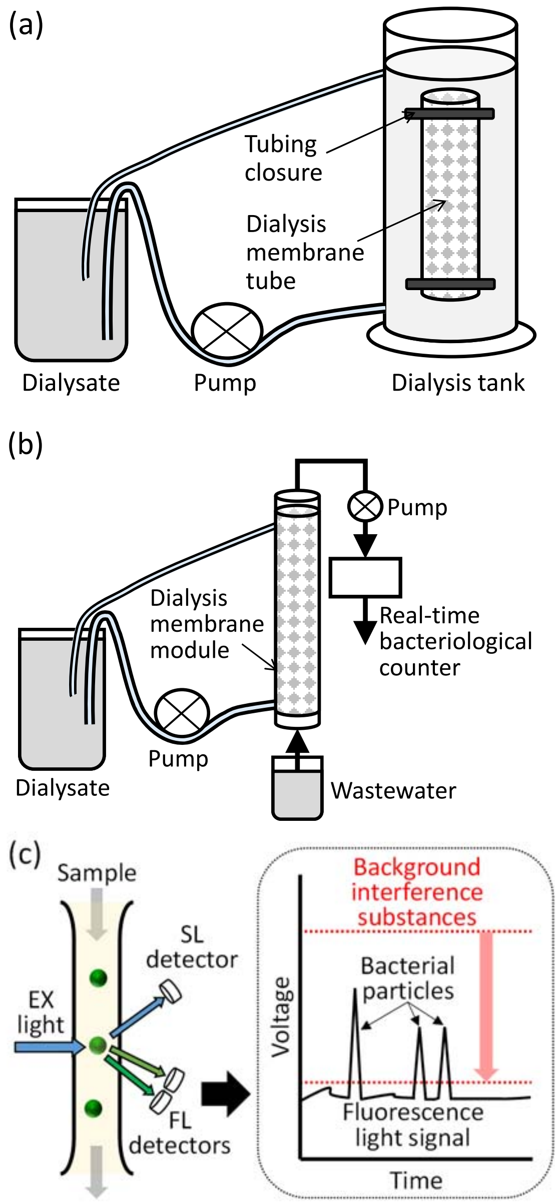

(a)
Tubing
closure
Dialysis
membrane
tube
Dialysate
Pump
Dialysis tank
(b)
Pump
Dialysis
membrane
module
Real-time
bacteriological
counter
Pump
Dialysate
Wastewater
(c)
Sample
SL
detector
EX
light
FL
detectors
Background
interference
substances
Bacterial
particles
Voltage
Fluorescence
light signal
Time

Fig. 1

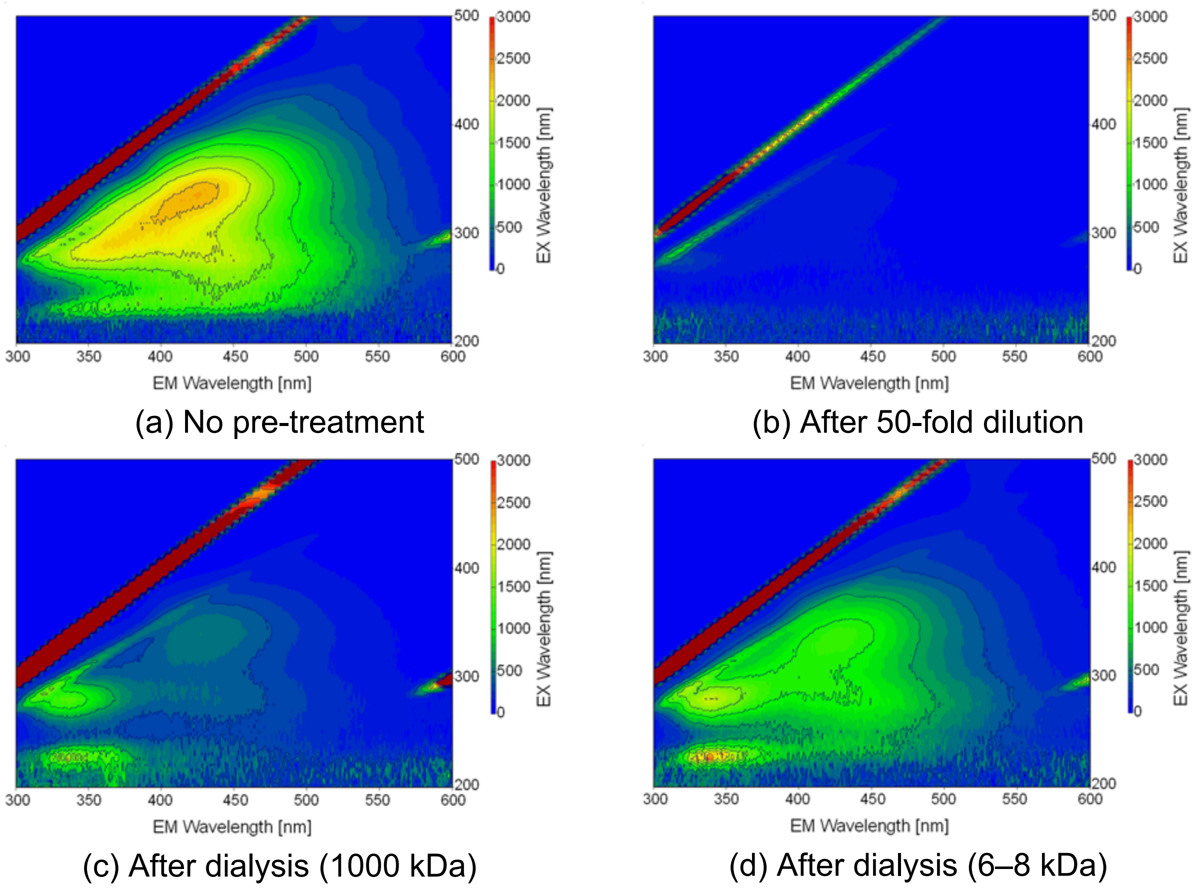
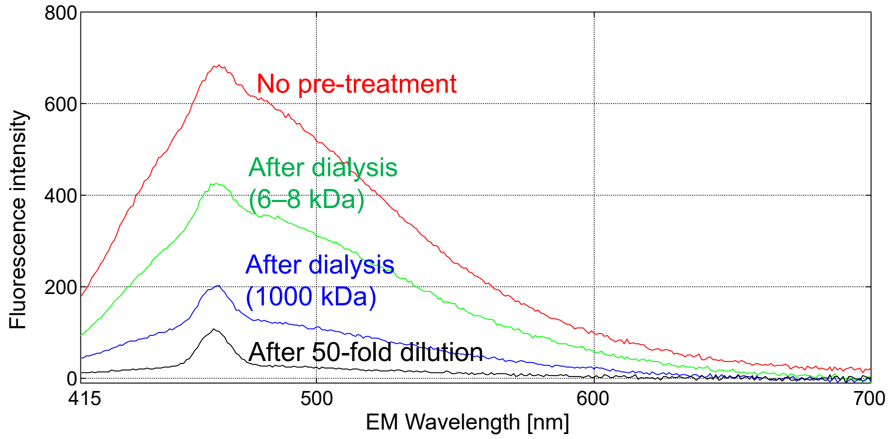
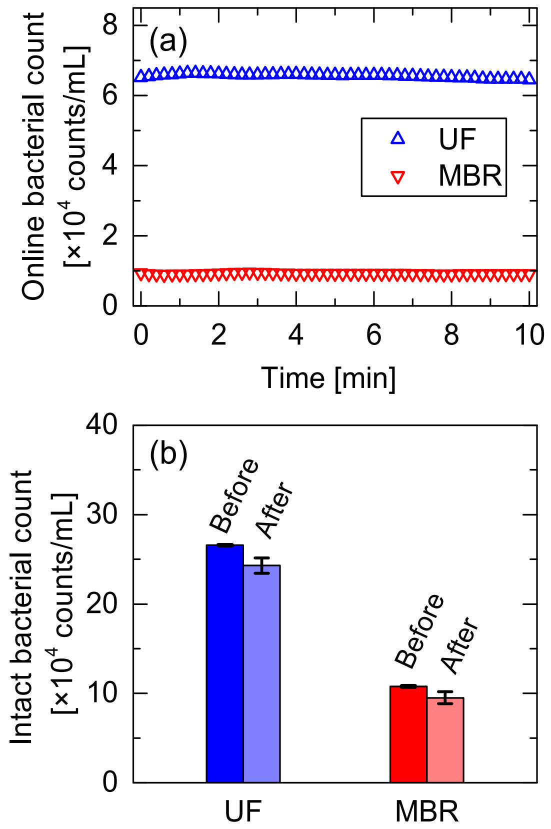

EM Wavelength [nm]
EX Wavelength [nm]
(a) No pre-treatment
(b) After 50-fold dilution
(c) After dialysis (1000 kDa)
(d) After dialysis (6–8 kDa)

Fig. 2



800
600
400
200
0
Fluorescence intensity
No pre-treatment
After dialysis
(6–8 kDa)
After dialysis
(1000 kDa)
After 50-fold dilution
415
500
600
700
EM Wavelength [nm]

Fig. 3



(a)
UF
MBR
Online bacterial count [×10^4 counts/mL]
Time [min]
(b)
Before
After
Before
After
Intact bacterial count [×10^4 counts/mL]
UF
MBR

Fig. 4