www.czasopisma.pan.pl PAN www.journals.pan.pl
Chemical and Process Engineering 2020, 41 (1), 69–76
DOI: 10.24425/cpe.2019.130224

STERILISATION OF NANOBUBBLE DISPERSIONS

Karol Ulatowski, Julia Fiuk, Paweł Sobieszuk*

Warsaw University of Technology, Faculty of Chemical and Process Engineering,
Waryńskiego 1, 00-645 Warsaw, Poland

In this paper, three methods of sterilisation are compared to determine their usability in nanobubble dispersion sterilisation: filtration, thermal sterilisation and sonication (in two systems: using a sonotrode and sonication bath). Nanobubble dispersions are most commonly generated in non-sterile systems which precludes them from use in most biological research. As a result of this study, filtration was chosen as the best method for nanobubble sterilisation.

Keywords: nanobubbles, nanodispersion, sterilisation, filtration, ultrasonication

1. INTRODUCTION

The term "nanobubbles" describes spherical gas domains in a liquid whose diameter does not exceed 1 μm (Tsuge, 2015; Ulatowski et al., 2019). Due to such diminutive size, nanobubbles are characterised by rising velocity of a magnitude similar or lower to Brownian motion (according to Stokes law) (Li et al., 2014) and surprisingly long lifetime in liquid (Duval et al., 2012; Oh and Kim, 2017; Ulatowski et al., 2019). Nanobubbles have been found useful in various branches of industry, including wastewater treatment and flotation (Calgaroto et al., 2014; Gurung et al., 2016; Temesgen et al., 2017; Wang and Xu, 2012), fuel enrichment (Oh et al., 2015) and surface cleaning (Zhu et al., 2016). However, the interactions of nanobubbles with living matter are especially interesting for us. Presently, the most common interaction of carbon dioxide or ozone nanobubbles with microorganisms is disinfection. Nanobubbles were used in inactivation of *E. coli* suspended in a saline solution using carbon dioxide nanobubbles (Kobayashi et al., 2009) and bubbles generated by ultrasonication in LB broth (Luu et al., 2019). Other studies report disinfection of plant roots using ozone nanobubbles (Kobayashi et al., 2011). On the contrary, the positive interaction of nanobubbles with living matter is also observed. Oxygen nanobubbles are reported to promote the growth of animals (mice, rainbow trout and sweetfish) (Ebina et al., 2013) and plants (*Brassica campestris*, lettuce) (Ebina et al., 2013; Park and Kurata, 2009). Oxygen nanobubbles were also used to increase the metabolic rate of L929 mice cell cultures. Mixing water nanodispersion of oxygen with a culture medium showed that even a 1% addition of nanodispersion significantly increased the metabolic rate of mice cells (Ulatowski et al., 2018). Additionally, there are numerous reports of carbon dioxide nanobubble effectiveness in diabetic ulcer treatment (Riyad and Al-omary, 2018; Shalan et al., 2015). We expect that the usability of nanobubbles in cultures of microorganism, animal or plant cells as well as medical applications will increase in not so distant future.

All the references quoted above stressed the importance of using sterile media. Unfortunately, most of commonly used nanobubble generation methods are hard to carry out in sterile conditions. Setups for

* Corresponding author, e-mail: Pawel.Sobieszuk@pw.edu.pl

hydrodynamic methods of generation have a complex structure which cannot be sterilised by most popular methods. In that case, nanobubble dispersion has to be sterilised after generation. As such, the influence of sterilisation on the size distribution of bubbles in the dispersion needs to be investigated. Specifically, the aim of this study was to determine whether previously generated nanobubbles were present in the liquid after sterilisation and whether their size changed during the process. This paper presents sterilisation results of nanodispersion using three methods which are commonly used in biological laboratories – thermal sterilisation in an autoclave, filtration using microbiological filters and ultrasonication using a sonotrode or ultrasonic bath. We performed sterilisation of nanodispersions of two process gases: oxygen (necessary for aerobic organisms) and nitrogen (which is an inert gas, which enables to investigate the influence of sole presence of this kind of nanoobjects on microorganism culture density and morphology).

2. MATERIALS AND METHODS

2.1. Generation of nanodispersions

Nanodispersions were generated in distilled water using a porous membrane module equipped with three ceramic cylindrical membranes (pore diameter 0.2 μm). Inside the membranes water was flowing and cutting off bubbles which were formed by a gas squeezing through the membrane. Gas pressure and water flowrate were equal to 2.2 bar and 130 dm^3/h, respectively. The dispersion generated in this way was stored in a tank which was also the source of the liquid phase. Generation was carried out for 1 hour and immediately after turning off the generation system. The dispersion was sampled for sterilisation and size distribution density measurement. This method of generation allows for generation of nanobubbles consisting of one, strictly defined gas.

2.2. Thermal sterilisation

Thermal sterilisation was carried out in an autoclave in 400 ml bottles for 25 min in 121 °C. Next, samples were analysed for size distribution density of nanobubbles and sterility of acquired dispersion was assessed.

2.3. Sterilisation by filtration

Filtration was carried out using syringes with cellulose filters with pore diameter of 0.22 μm. To ensure the repeatable conditions of filtration, the process was carried out using a syringe pump. The flowrate of filtrate was set at 1.5 ml/min. Each filter was used three times and each filtration charge generated 3 distinct samples.

2.4. Sterilisation by ultrasonication

Two kinds of ultrasonic systems were used in this study to check two values of sonication frequencies, as according to literature the regions for nanobubble generation and sterilisation hardly overlap. The first system involved an ultrasonic homogeniser UP100H (Hielscher) and sonicated 100 ml of sample (nanodispersion of gas) in a plastic container using an MS3 sonotrode at a fixed sonication frequency of 20 kHz. The amplitude of sonotrode oscillation was changed and sonication lasted for 20 minutes. Three values of amplitude were chosen: 36 μm, 108 μm and 180 μm. The second system sonicated 400 ml of nanodispersion in a glass bottle at a fixed frequency of 40 kHz using a Sonic-14 (Polsonic) ultrasonic bath. Three durations of sonication were investigated: 20 min, 40 min and 60 min.

2.5. Size distribution density measurement

To obtain size distribution density data, a Malvern Zetasizer NanoZS was used. The measurement technique was Dynamic Light Scattering (DLS). Using the results of number distribution densities of nanobubble diameter the Sauter diameter d_{32} was calculated according to the formula

$$d_{32} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2}$$

where n_i is the number fraction of nanobubbles with diameter d_i.

2.6. Sterility assessment

From each sample after generation, 0.5 ml was transferred onto Czapek agar or nutrient agar plates and incubated for 48 h at 25 °C. Next, a picture of each plate was taken and sterility was visually assessed. We used two kinds of microbiological solid media to observe the growth of a wider spectrum of microbes, especially microfungi on Czapek agar, which are the most common source of biological contamination. Two technical replicates of each sample on each solid medium were prepared.

3. RESULTS AND DISCUSSION

3.1. Nanodispersion after generation

The sterility assessment has shown that before sterilisation there were microorganisms in the dispersion, so the dispersion was not sterile. There was a significant difference between the morphology of the colonies of microorganisms cultured on nutrient agar from oxygen and nitrogen nanodispersions. The morphology of cultures on Czapek agar did not differ. CFU originating from oxygen nanodispersion was much higher than that of nitrogen nanodispersion on both media.

3.2. Autoclave sterilisation

Autoclaving obviously sterilised the dispersion of nanobubbles. However, bubbles were not preserved, as DLS results did not present repetitive results even for the same sample. For that reason, autoclaving is not a good method for nanobubble dispersion sterilisation. This result was expected as the drastic increase of temperature caused high reduction of solubility of gases. That led to destruction of nanobubbles present in the liquid.

3.3. Sterilisation by filtration

Filtration allowed us to sterilise dispersions, as no colonies (CFU equals zero) were seen on Petri plates after filtration. Even after one filtration all microorganisms were removed from the volume of the media. As such, no pictures of the plates are given, as they carry no additional information. Filtration was similarly expected to sterilise the dispersion but the main scientific interests were whether the average nanobubble diameter changed during filtration and between consequent filtrations. Figure 1 presents the Sauter diameter of nanobubbles after generation and after consequent filtrations. The diameter changed significantly and

the Sauter diameter decreased during filtration from ~ 400 nm to ~ 200 nm, i.e. the diameter of bubbles after filtration corresponded to the pore size (220 nm). The size of bubbles did not change after second and third filtration. Thus, filtration proved to be a reliable method for sterilisation of media which also preserved the existence of nanobubbles in dispersion. The restriction of this method is low value of efficiency of filtration using membranes with 0.22 μm pores.

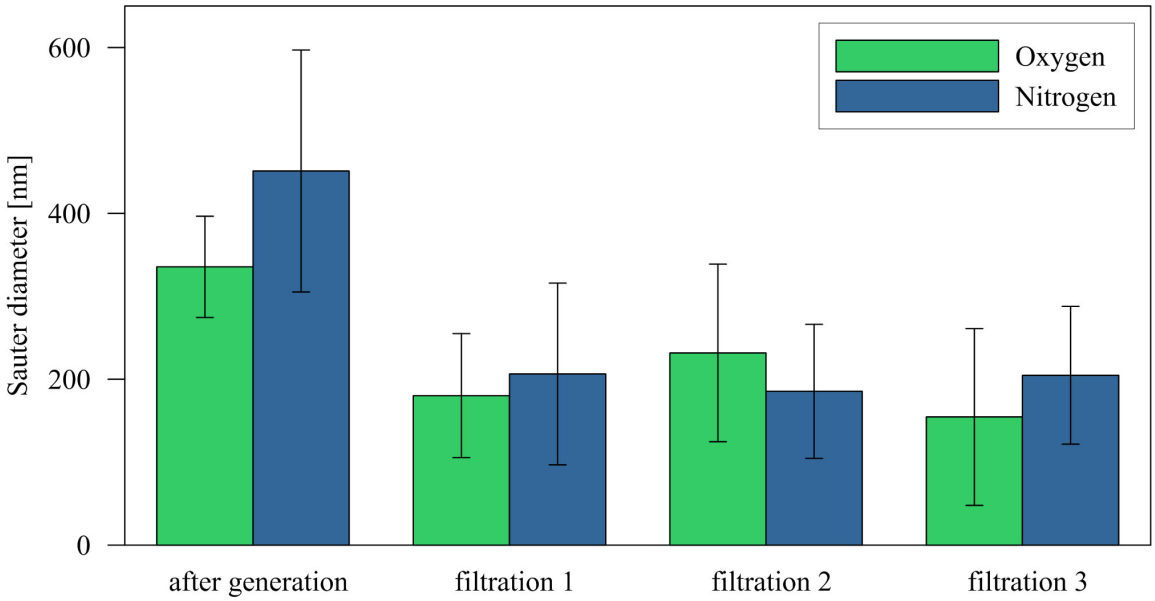

Fig. 1. Sauter diameter of bubbles in dispersion of nitrogen and oxygen nanobubbles before and after filtration

3.4. Sterilisation by sonication

Sonication is reported as both the sterilisation method (Bałdyga et al., 2018) and nanobubble generation method (Cho et al., 2005; Luu et al., 2019), but the regions of frequencies which allow for the presence of these effects hardly overlap. As shown in this study and in the literature, some microorganisms can be disintegrated even by frequencies which should generate (or at least preserve) nanobubbles, but the concurrent presence of both phenomena is not common. Other research teams have seen this problem and used a different method for sterilisation of medium and nanobubbles were generated only in a sterile medium. Luu et al. (2019) investigated the influence of nanobubbles on *Escherichia coli* growth rate (Luu et al., 2019). The sterile (autoclaved) medium was treated with ultrasonication in sterile conditions to generate nanobubbles. The resulting medium with nanobubbles was used as a culture medium for bacterial growth. However, neither the size of generated bubbles nor the actual proof were given. The main problem with this approach is that without complex (and hard to sterilise and keep sterile) systems, one can only generate bubbles of air. For generation of nanobubbles of different gases, one needs to keep the liquid saturated with a gas, which is not always possible to couple with a sonication system. On the other side of the spectrum there are hydrodynamic methods, which generate non sterile dispersion of any gas needed. In our case the medium generated by the hydrodynamic method was treated with ultrasonication to sterilise the dispersion and check the preservation of bubbles in the liquid.

The first batch of samples was sonicated using a UP100H ultrasonic homogeniser with an MS3 sonotrode and a frequency of 20 kHz. Sterility assessment has shown that this method of sonication was not sterilising the dispersion as the growth of microorganism was visible on both nutrient agar and Czapek agar. However, higher amplitude values did destroy most of microorganisms which were able to grow on Czapek agar. Nevertheless, the colonies were barely visible which may indicate that the vegetative forms of microorgan-

isms were killed, while the spores were preserved. According to Baldyga et al. (2005), a sonotrode with 20 kHz of frequency was able to disintegrate *Saccharomyces cerevisiae* cells (Bałdyga et al., 2018). This microorganism would grow on Czapek agar and its cells could be disintegrated by sonication even at low frequencies.

As one can see in Fig. 2, bubbles were preserved in the dispersion and their diameter hardly changed for 36 μm and 108 μm of amplitude compared to the diameter observed before sterilisation. However, for 180 μm of amplitude, the bubble diameter decreased significantly and standard deviation was much lower. That may lead to the conclusion that for this set of parameters, only smaller bubbles were preserved, while larger ones coalesced and rose to the free surface of the liquid. As such, these parameters of sonication did not sterilise the dispersion, but allowed for preservation of bubbles. That fact confirms the literature reports that state that frequencies under 20 kHz do not destroy nanobubbles and can even generate additional nanobubbles by cavitation from gases dissolved in a liquid (Cho et al., 2005).

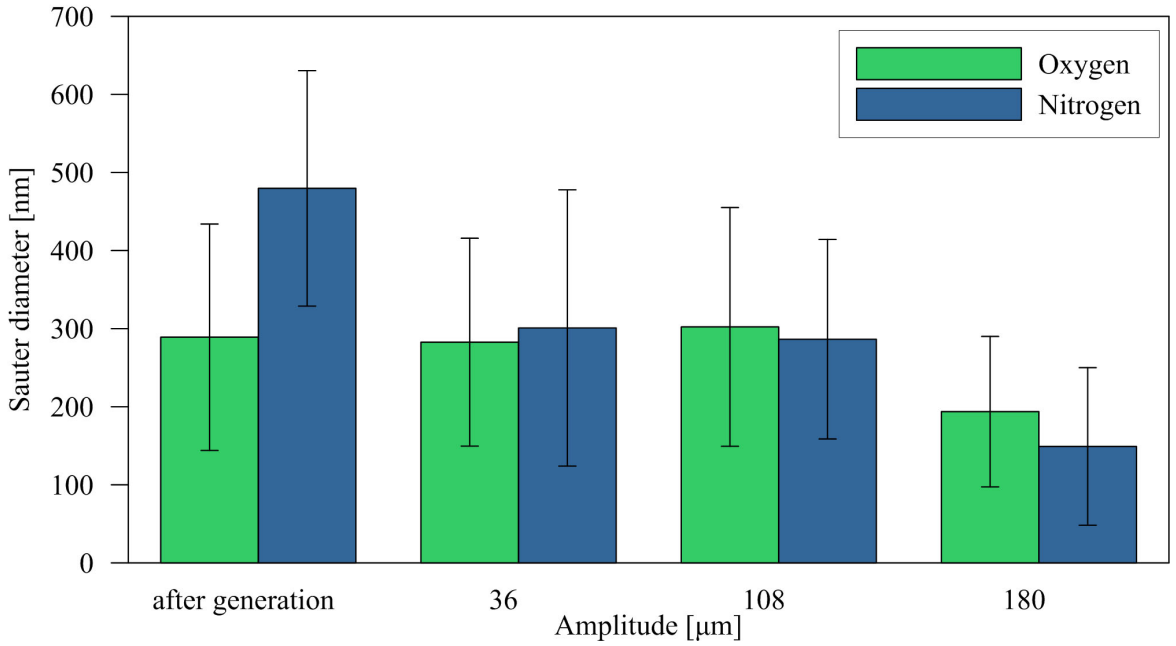

Fig. 2. Sauter diameter of nanobubbles after generation and after sonication using a sonotrode

As the main goal of this work was to achieve sterile dispersion of nanobubbles, a different approach was investigated. The frequency of 40 kHz is commonly used for cell disintegration (Tiehm et al., 2001) and therefore a sonication bath with the set frequency of 40 kHz was used. Sonication lasted for 60 minutes and sampling was carried out in 20^{th} and 40^{th} minute of sonication in addition to sampling after sonication. Even in a sample acquired after 20 minutes of sonication, no colonies of microorganisms were visible on either nutrient or Czapek agar.

Unfortunately, as shown in Fig. 3, extremely high values of standard deviation which are increasing with sonication time are implying that this method is not ideal for nanobubble dispersion sterilisation. Although the values of Sauter diameter after 20 minutes of sonication are lower than 1000 nm (and bubbles of this diameter can be called nanobubbles), the result is near this boundary. The value of Sauter diameter grew in time and one cannot be sure whether more resistant microorganisms would be disintegrated in this time. Because of drastic changes in Sauter diameter, this method of sterilisation is not suitable for sterilisation of nanobubble dispersions despite the fact that sterility was achieved.

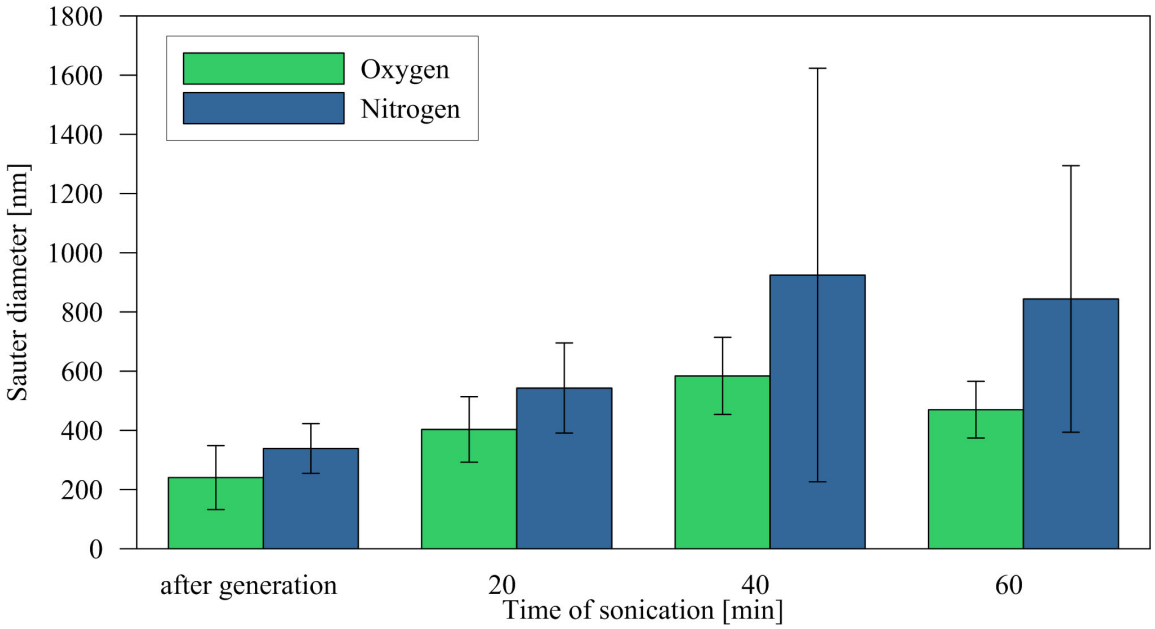

Fig. 3. Sauter diameter of nanobubbles after generation and after sonication using sonication bath

4. CONCLUSIONS

In this paper the problem of obtaining sterile nanodispersions of nitrogen or oxygen in water was considered. This is an important issue, because we predict an increasing use of nanodispersions in various cultures. A solution has been proposed in which a dispersion is produced in a flowing system with a membrane and sterilised afterwards. In such a generation system it is possible to obtain a nanodispersion from a strictly defined gas, i.e. one that is pressurised through a membrane. For the second stage (sterilization) of our study, the process was carried out with three methods: in an autoclave, as a result of sonication and filtration.

As predicted, the process of thermal sterilization destroyed all vegetative forms and spores of microorganisms, but caused a total destruction of nanodispersion.

The sonication process was carried out at two frequency values: 20 kHz (using a sonotrode) and 40 kHz (using a sonication bath). The first value is a typical upper boundary of nanodispersion production. The second value is typical for sterilization by sonication. Since both frequency values are different, it was not possible to combine the two phenomena, which would seem to be very beneficial. The use of 20 kHz did not destroy nanobubbles, but also did not sterilise nanodispersions. On the other hand, the use of frequency values of 40 kHz excellently sterilised nanodispersions, but at the same time completely destroyed nanobubbles. It is possible to produce a sterile nanodispersion by sonication of a sterile liquid, but it is nearly impossible to define the gas from which nanodispersions are generated.

The last tested method, i.e. the filtration of nanodispersion with 0.22 μm filters, proved to be appropriate. Sterile solutions with still, stable and suspended nanobubbles were obtained.

Finally, in order to obtain sterile nanodispersions where bubble size is preserved, they should be first produced and then sterilised using filtration.

This work was supported by the National Science Centre, Poland, grant number 2018/29/B/ST8/00365.

SYMBOLS

d_i nanobubble diameter, m
d_{32} Sauter diameter defined by Eq. (1), m

REFERENCES

Bałdyga J., Jasińska M., Dzięgielewska M., Żochowska M., 2018. Effect of sonication reactor geometry on cell disruption and protein release from yeast cells. *Chem. Process Eng.*, 39, 475–489. DOI: 10.24425/cpe.2018.124973.

Calgaroto S., Wilberg K.Q., Rubio J., 2014. On the nanobubbles interfacial properties and future applications in flotation. *Miner. Eng.*, 60, 33–40. DOI: 10.1016/j.mineng.2014.02.002.

Cho S.H., Kim J.Y., Chun J.H., Kim J.D., 2005. Ultrasonic formation of nanobubbles and their zeta-potentials in aqueous electrolyte and surfactant solutions. *Colloids Surf., A*, 269, 28–34. DOI: 10.1016/j.colsurfa.2005.06.063.

Duval E., Adichtchev S., Sirotkin S., Mermet A., 2012. Long-lived submicrometric bubbles in very diluted alkali halide water solutions. *Phys. Chem. Chem. Phys.*, 14, 4125. DOI: 10.1039/c2cp22858k.

Ebina K., Shi K., Hirao M., Hashimoto J., Kawato Y., Kaneshiro S., Morimoto T., Koizumi K., Yoshikawa H., 2013. Oxygen and air nanobubble water solution promote the growth of plants, fishes, and mice. *PLOS ONE*, 8 (6), e65339. DOI: 10.1371/journal.pone.0065339.

Gurung A., Dahl O., Jansson K., 2016. The fundamental phenomena of nanobubbles and their behavior in wastewater treatment technologies. *Geosyst. Eng.*, 19 (3), 133–142. DOI: 10.1080/12269328.2016.1153987.

Kobayashi F., Hayata Y., Ikeura H., Tamaki M., Muto N., Osajima Y., 2009. Inactivation of E*sherichia coli* by CO_2 microbubbles at a lower pressure and near room temperature. *Trans ASABE*, 5 2(5), 1621–1626.

Kobayashi F., Ikeura H., Ohsato S., Goto T., Tamaki M., 2011. Disinfection using ozone microbubbles to inactivate *Fusarium oxysporum* f. sp. melonis and *Pectobacterium carotovorum subsp. carotovorum*. *Crop Prot.*, 30, 1514–1518. DOI: 10.1016/j.cropro.2011.07.018.

Li H., Hu L., Song D., Lin F., 2014. Characteristics of micro-nano bubbles and potential application in groundwater bioremediation. *Water Environ. Res.*, 86, 844–851. DOI: 10.2175/106143014X14062131177953.

Luu T.Q., Hong Truong P.N., Zitzmann K., Nguyen K.T., 2019. Effects of ultrafine bubbles on gram-negative bacteria: Inhibition or selection? *Langmuir*. DOI: 10.1021/acs.langmuir.9b02641.

Oh S.H., Han J.G., Kim J.M., 2015. Long-term stability of hydrogen nanobubble fuel. *Fuel*, 158, 399–404. DOI: 10.1016/j.fuel.2015.05.072.

Oh S.H., Kim J.M., 2017. Generation and stability of bulk nanobubbles. *Langmuir*, 33, 3818–3823. DOI: 10.1021/acs.langmuir.7b00510.

Park J.S., Kurata K., 2009. Application of microbubbles to hydroponics solution promotes lettuce growth. *HortTechnology*, 19, 212–215. DOI: 10.21273/HORTSCI.19.1.212.

Riyad G., Al-Omary H., 2018. Physiological effects of carbon dioxide treatment on diabetic foot ulcer physiological effects of carbon dioxide treatment on diabetic foot ulcer patients. *IOSR J. Pharm. Biol. Sci.*, 13 (5) 1–7. DOI: 10.9790/3008-1305040107.

Shalan N., Al-Bazzaz A., Al-Ani I., Najem F., Al-Masri M., 2015. Effect of carbon dioxide therapy on diabetic foot ulcer. *J. Diabetes Mellitus*, 5, 284–289. DOI: 10.4236/jdm.2015.54035.

Temesgen T., Bui T.T., Han M., Kim T.L, Park H., 2017. Micro and nanobubble technologies as a new horizon for water-treatment techniques: A review. *Adv. Colloid Interface Sci.*, 246, 40–51. DOI: 10.1016/j.cis.2017.06.011.

Tiehm A., Nickel K., Zellhorn M., Neis U., 2001. Ultrasonic waste activated-sludge disintegration for improving anaerobic stabilization. *Water Res.*, 35, 2003–2009. DOI: 10.1016/s0043-1354(00)00468-1.

Tsuge H., 2015. *Micro- and nanobubbles. Fundamentals and applications*. Boca Raton: Pan Stanford Publishing, Boca Raton.

Ulatowski K., Sobieszuk P., Kuźmińska A., Ciach T., 2018. Investigation of cytotoxicity of oxygen nanobubble dispersion in water. *Acta Scientiarum Polonorum*, 17 (2), 51–58. DOI: 10.30825/5.biot.58.2018.17.2 (in Polish).

Ulatowski K., Sobieszuk P., Mróz A., Ciach T., 2019. Stability of nanobubbles generated in water using porous membrane system. *Chem. Eng. Process. Process Intensif.*, 136, 62–71. DOI: 10.1016/j.cep.2018.12.010.

Wang J.L., Xu L.J., 2012. Advanced oxidation processes for wastewater treatment: Formation of hydroxyl radical and application. *Crit. Rev. Env. Sci. Technol.*, 42, 251–325. DOI: 10.1080/10643389.2010.507698.

Zhu J., An H., Alheshibri M., Liu L., Terpstra P.M.J., Liu G., Craig V.S.J., 2016. Cleaning with bulk nanobubbles. *Langmuir*, 32, 11203–11211. DOI: 10.1021/acs.langmuir.6b01004.

Received 30 January 2020
Received in revised form 15 February 2020
Accepted 11 March 2020