Comment on 'UV Disinfection Induces a VBNC State in *Escherichia coli* and *Pseudomonas aeruginosa*'

Ernest R. Blatchley III, Ph.D., P.E. BCEE, F. ACSE[1*], Kumiko Oguma, Ph.D.[2] and Regina Sommer, Ph.D., MEng.[3]

1. Professor, Lyles School of Civil Engineering and Division of Environmental & Ecological Engineering, Purdue University, West Lafayette, IN, USA
2. Associate professor, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan
3. Professor, Institute for Hygiene and Applied Immunology Water Hygiene, Medical University-Vienna, Vienna, Austria
*Corresponding author: Ernest R. Blatchley III (blatch@purdue.edu)

A recently published paper (Zhang et al. 2015) reported on induction of a viable but nonculturable (VBNC) state among populations of *E. coli* and *P. aeruginosa*. The authors used this claim and other information from their paper to reach a conclusion that "A public health risk may exist when UV radiation is utilized as a drinking water treatment system."

We believe the authors have greatly overstated the significance of their work. Moreover, there are numerous fundamental flaws in the literature review, experimental methods, and interpretations of their data. As a result, we do not believe the central conclusion of this paper (described above) to be scientifically defensible.

The VBNC state was first reported by Xu et al. (1982) in what now has become a classic paper. VBNC bacteria are in a dormant state which allows them to retain metabolic activity, while failing to grow on routine bacteriological media (Oliver 2005). The morphology, physiological, and phenotypic characteristics of VBNC bacteria may change relative to unaffected cells. As many as 51 species of bacteria have been reported to exist in a VBNC state (Li et al. 2014). Induction of the VBNC state is considered to be a common response of bacteria to exogenous stress; of particular relevance to water treatment is induction by commonly used disinfectants, including chlorine (free and combined), ozone, and ultraviolet radiation.

Zhang et al. (2015) suggest their work is the first to demonstrate UV induction of the VBNC state. In fact, this behavior has been reported many times previously and is widely recognized among researchers in the area as an attribute of UV disinfection. As an example, consider the data presented in Table 1, which summarizes responses of laboratory-grown cultures of bacteria to UV_{254} irradiation under a collimated beam device. For the three species of bacteria investigated, UV_{254} doses that achieved 6-7 $\log_{10}$ units of reduction in the concentration of culturable bacteria yielded reductions of respiration (measured directly using a respirometer) of only about 75% (i.e., roughly 0.6 $\log_{10}$ unit reduction). These vast differences in culturability and respiration provide a clear indication of the development of VBNC bacteria. Said et al. (2010) also reported induction of the VBNC state by examining infection of UV_{254}-irradiated *E. coli* by Qβ phage. Their results demonstrated loss of culturability among bacteria that retained their susceptibility to phage infection.

Table 1. Summary of culturability and respiration rate responses to UV_{254} irradiation from a collimated beam. Reported culturability (N) and respiration rate (R) responses are normalized with respect to their values for non-irradiated cultures (N_0 and R_0, respectively). Data from Blatchley et al. (2001).

Bacterium	UV_{254} Dose (mJ/cm^2)	N/N_0	R/R_0
E. coli	100	$10^{-6.8}$	0.23
P. aeruginosa	50	n/a	0.26
S. faecalis	70	$10^{-6.0}$	0.25

Recent history has demonstrated the importance of proper interpretation of microbial responses to UV radiation, as well as proper interpretation of the results of assays used to measure these responses. Until the late 1990s, the consensus opinion within the scientific community was that UV irradiation was ineffective for control of protozoan parasites, including *Cryptosporidium parvum* and *Giardia lamblia*.

This erroneous conclusion was attributable to the use of analytical methods that were inconsistent with the mechanism of disinfection that is associated with UV-based processes. The works of Bukhari et al. (1999), Craik et al. (2000), and many others indicated that when appropriate analytical methods are used (i.e., methods based on infectivity, rather than indirect measures of the potential for infection), UV emerged as the disinfectant of choice for control of these protozoan para-

sites. The implications of this lesson should not be lost when investigating the responses of other microorganisms to UV irradiation.

Information from the refereed literature also indicates that other commonly-used disinfectants, including chlorine, will induce the VBNC state. For example, Dukan et al. (1997) demonstrated that exposure of *E. coli* to free chlorine will yield three subpopulations: dead cells, culturable cells, and VBNC cells. Subsequent exposure of the bacterial culture to nutrient-free phosphate buffer resulted in regrowth. They provided evidence that much of the population recovery was attributable to regrowth of the culturable cells in the population. However, they also provided information to indicate that resuscitation of VBNC cells contributed to observed regrowth. A subsequent study by Rockabrand et al. (1999) demonstrated chlorine-induction of VBNC cells within a culture of *E. coli*. Their investigation was based on presence or absence of proteins that are included or excluded, respectively from cultures of growing bacteria.

Given the widely-held view that the VBNC state is a common response of bacteria to exogenous stresses (Li et al. 2014), it is not surprising that disinfectants, such as UV radiation and chlorine, would elicit this response. The work of Zhang et al. (2015) supports previous publications that have provided evidence of VBNC induction by UV radiation. The new information provided by Zhang et al. (2015) relates largely to their application of molecular biology to characterize the VBNC response, and they are to be commended for their use of these tools to examine the VBNC phenomenon. However, some aspects of their experiments do not to conform to well-established methods for conducting UV exposures of microbial populations.

Zhang et al. (2015) used a UV source that involved a vertically-oriented, low-pressure Hg lamp surrounded by a lampshade. At the very least, this represents an unconventional configuration for a UV source to be used as a "collimated beam." There were no data provided in the paper to indicate beam uniformity or calculations of the so-called "petri factor," as defined by Bolton and Linden (2003). More generally, the definition of UV dose used in their investigation is not provided. By inference, it appears that the "dose" values reported by Zhang et al. (2015) represent the product of incident irradiance and exposure time.

In other words, it appears that the authors have failed to account for the various terms that are needed to accurately describe the UV dose that is received by a suspension of microorganisms in a collimated beam experiment. These correction factors are described in great detail in the paper by Bolton and Linden (2015) and by the Austrian Standards Institute (ÖNORM M 5873-1:2001), which now represent standards for collimated beam testing.

To be fair, some of the correction factors described by Bolton and Linden (2003) result in small changes in the estimate of the UV dose delivered in a collimated beam experiment, at least when other conditions of proper experimentation are used (see below). However, another important flaw in the methods used by Zhang et al. (2015) appears to have contributed to considerable error in the interpretation of their data. Specifically, they report the use of bacterial concentrations of approximately 2 x 10^9 CFU/mL. This concentration is two to three orders of magnitude higher than used in most previously published work involving collimated beam exposures of bacterial cultures; the use of such a high bacterial concentration likely will lead to aggregation of bacteria (turbidity) and low UV transmittance. Both of these attributes will shield bacteria from UV exposure and reduce the UV dose actually received by the cells. Zhang et al. (2015) provided no indication that these attributes of their experiments were accounted for in their calculations of UV dose.

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This last point is important with respect to the presentation in Zhang et al. (2015) of changes in bacterial culturability as a function of UV dose. Their data, which were provided for *E. coli* and *P. aeruginosa*, differ markedly from previously published reports of changes in culturability that occur among suspensions of vegetative bacterial cells. For comparison, Figure 1 provides a summary of data from two previous investigations, along with the data provided by Zhang et al. (2015) for responses of *E. coli*. Included in this figure is a data set from Blatchley et al. (2001) that demonstrates the tailing behavior also described by Zhang et al. (2015). The tailing behavior in the data set from Blatchley et al. (2001) was attributed to formation of bacterial aggregates, which were confirmed by microscopy. Sommer et al. (2000) provided data to summarize the changes in culturability among eight strains of *E. coli*, which showed considerable variability. Included in Figure 1 are the dose-response data for the most (O157:H7) and least (O25:K98:NM) UV-sensitive of the *E. coli* suspensions investigated by Sommer et al. (2000). The axis scales were selected for Figure 1 so as to allow for direct comparison of the responses of various *E. coli* cultures to UV_{254} irradiation.

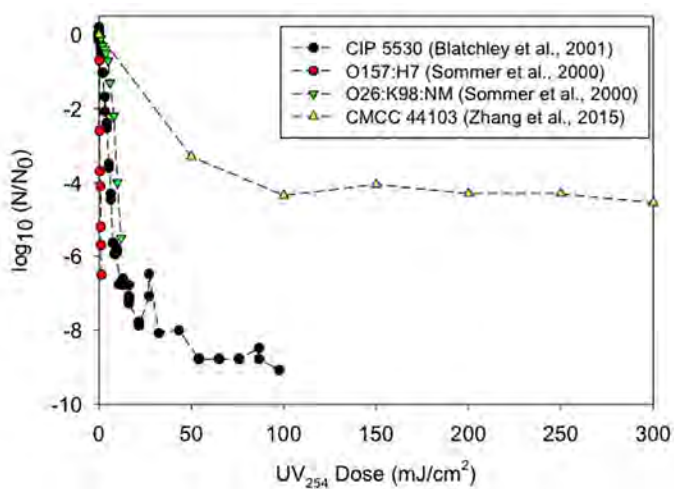

Figure 1. Comparison of reported changes in *E. coli* culturability in response to UV_{254} irradiation. Labels in the legend indicate the strain of *E. coli*; parenthetical entries identify the reference from which the data were taken.

The data of Zhang et al. (2015) indicate apparent UV resistance that is at least an order or magnitude greater than any of the other data sets included in this figure, or that can be found in the refereed literature. More generally, Chevrefils et al. (2006) assembled a comprehensive review of published UV dose-response data for a wide range of microorganisms. Among the vegetative bacteria included in their summary, the maximum UV_{254} dose required to achieve a 5 $\log_{10}$ unit reduction in culturability [roughly the location of the tailing behavior reported by Zhang et al. (2015)] was 14 mJ/cm^2. In the data provided by Zhang et al. (2015), a dose of at least 100 mJ/cm^2 was required to approach 5 $\log_{10}$ units of decrease in *E. coli* culturability.

Zhang et al. (2015) also included data to describe changes in culturability of *P. aeruginosa* in response to UV_{254} irradiation. Very little data are available in the literature to describe the response of *P. aeruginosa* to UV_{254} irradiation, probably because this bacterium tends to be "sticky" and forms aggregates quite easily. Aggregation greatly complicates the interpretation of bacterial responses to UV irradiation because it provides a mechanism for protection of bacterial cells from UV exposure. When the potential for aggregation exists in an experiment, it needs to be accounted for in the analysis and interpretation of the resulting data. No such analysis was included in the paper by Zhang et al. (2015).

Given the general observation that vegetative cells of bacteria tend to be quite sensitive to UVC radiation, it seems unlikely that the data reported by Zhang et al. (2015) are an accurate portrayal of the actual UV dose-response behavior of their bacteria. More plausible explanations for the large differences between the sensitivity to UVC radiation in their study and previous reports of this behavior are the flaws in the experimental design described above.

To clarify the impact of the experimental design used by Zhang et al. (2015), we performed UV_{254} irradiation experiments with *P. aeruginosa* in suspensions of two different concentrations. *P. aeruginosa* (NCTC 10662; obtained from DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was grown in LB broth for 16 h at 37°C, washed by centrifugation, and resuspended in sterile saline as described by Zhang et al. (2015). One suspension of *P. aeruginosa* was adjusted to a concentration of about 1 x 10^6 CFU/mL, the other to a concentration of 1 x 10^9 CFU/mL based on colony count using nutrient agar (Columbia agar base, Oxoid, UK; incubation 37°C for 24 h; pour plate method). Twenty mL portions of the suspensions each were irradiated in sterile petri dishes under continuous stirring on a magnetic stirrer in a laboratory irradiation device according to ÖNORM M 5873-1:2001 (Austrian Standards Institute, 2001). The concentrations of *P. aeruginosa* in the non-irradiated and irradiated samples were quantified in triplicate by means of the same pour plate method as described above. The UV_{254} transmittance (1.0 cm; UV-VIS spectrophotometer, Lambda 25, Perkin Elmer, USA) and the turbidity (nephelometer, Turb 430 IR, WTW, Germany) of the suspensions were measured. The results are given in Table 2.

Table 2. UV_{254} transmittance (1.0 cm) and turbidity of the two *P. aeruginosa* suspensions used for the UV_{254} irradiation experiments.

P. aeruginosa concentration (CFU/mL)	UV_{254} transmittance (1.0 cm) (%)	Turbidity (NTU)
1 x 10^6	83.0	0.89
1 x 10^9	1.7	552

In Figure 2 a picture of the suspensions of *P. aeruginosa* in the sample flasks is shown. Even with the naked eye the huge difference in turbidity caused by the two different bacterial concentrations can be seen.

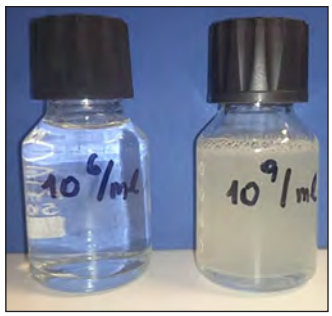

Figure 2. The suspensions of *P. aeruginosa* with two different concentrations (1 x 10^6 CFU/mL and 1 x 10^9 CFU/mL).

In Figure 3 the results of the UV_{254} inactivation curves of *P. aeruginosa* in the suspensions with two different concentrations are presented. The impact of the protective effect due to the low UVT_{254} and high-turbidity in the suspension with a bacterial concentration of 1 x 10^9 CFU/mL can be seen clearly.

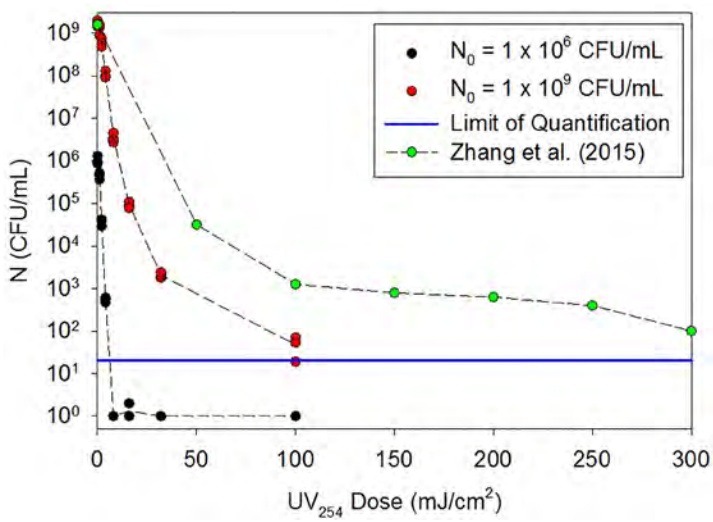

Figure 3. UV_{254} inactivation of *P. aeruginosa* in suspensions with two different concentrations (N_0) performed in a laboratory irradiation device according to ÖNORM M 5873-1:2001.

Zhang et al. (2015) did not account for UV_{254} transmittance when calculating the UV fluence (dose). Moreover, the authors used the nominal lamp emission given by the manufacturer as irradiance (0.28 mW/cm²) and multiplied it by the UV irradiation time. No measurements of the actual irradiance were performed, and the irradiance was not even corrected for the distance between lamp and sample (33 cm). The extraordinarily high UV_{254} resistance of *P. aeruginosa* as presented by Zhang et al. (2015) can be attributed to these non-regular circumstances and has to be seen as an experimental artifact.

Since turbidity compromises the reliability of disinfection processes (chlorine, ozone and UV irradiation) strict limits have been implemented in international regulations for drinking water. The World Health Organization (WHO) recommends for drinking water, especially when disinfection is in place, values for turbidity of 0.1 to 1 NTU (WHO, 2006). From the data given in Table 2, it can clearly be seen that at a bacterial concentration of 1 x 10^9 CFU/mL, the turbidity (552 NTU) was far beyond the maximum acceptable limit for regular disinfection conditions, and data gained under such circumstances cannot be seen as valid. Suspensions with a bacterial concentration of up to 1 x 10^6 CFU/mL ensure correct conditions to obtain reliable UV inactivation data.

From a public health standpoint, an important issue associated with VBNC cells is the potential for them to regain culturability, a process often described in the literature as resuscitation. Zhang et al. (2015) reported the ability of UV-induced VBNC cells to resuscitate by using a technique of dilution of UV_{254}-irradiated samples to a concentration well below 1 CFU per culture tube. This strategy, which has been used in many investigations of resuscitation of VBNC cells (Oliver 2005; Li et al. 2014), is applicable when the concentration of VBNC cells in the original (undiluted) sample is higher than the concentration of culturable cells.

In the case of Zhang et al. (2015), they used the appearance of turbidity during incubation of diluted samples as an indication of resuscitation. A heterotrophic plate count (HPC) growth medium was used in these experiments, which is consistent with the understanding that VBNC cells may not respond to selective growth media. However, there was no recognition of the potential for interference from other bacteria, a common problem in the application of HPC methods. There was no confirmation that the appearance of turbidity in their experiments was attributable to the target bacteria.

A related issue is the potential for bacterial recovery by dark repair or photoreactivation. These processes represent well-

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known mechanisms for recovery of culturability among UV-damaged cells (Jagger 1967; Oguma et al. 2002). No information is provided in the paper to indicate an investigation of dark repair or photoreactivation, both of which could be viewed as forms of resuscitation.

Lastly, the authors state that "In Europe, UV radiation has been applied since the 1980s for the disinfection of drinking and reclaimed water to reduce heterotrophic plate counts," and they refer to Kruithof et al. (1992) and Seckmeyer et al. (1994) to support this statement.

However, this statement is incorrect, since (1) in Europe, UV radiation has been used for many years for drinking water disinfection and not only for reducing the concentration of HPC, and (2) Seckmeyer et al. (1994) describe changes of UV-B irradiation in the environment due to clouds, aerosols and stratospheric ozone; their paper has no connection to UV disinfection of drinking water.

Induction of the VBNC state is an important consideration in the application of any disinfection process, including UV. Zhang et al. (2015) are to be commended for inclusion of molecular biological methods in the examination of this phenomenon, as related to UV disinfection. However, we encourage them to seek guidance from experts in the field regarding the conduct of bench-scale UV exposure experiments. Lastly, we feel that existing scientific information indicates that when properly applied, UV disinfection does not introduce a public health risk, as suggested by Zhang et al. (2015). Quite the contrary: UV disinfection has been demonstrated to represent an effective strategy for reduction of risk associated with microbial pathogens in water supplies. ■

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