

## **Advanced oxidation process based water disinfection- the microbiology beyond bacterial inactivation**

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**Abstract**

Different water treatment regimens are revealed to have potential in enriching antibiotic resistant bacteria (ARB). Advanced oxidation processes (AOPs) based disinfection techniques have been studied widely in the recent times due to their advantages over conventional treatment methods. However, bacterial response and adaptations against the hostile environments of AOPs is not clearly understood yet. Based on the existing knowledge on the ways in which bacteria surpass the antibiotic treatment, here we propose few important aspects of bacterial adaptation which could be true for AOPs as well since both antibiotics and AOPs generate reactive oxygen species (ROS) during their modes of action. We discuss the plausible role of ROS in the selection of ARB and bacterial heterogeneity as a strategy to bypass the lethal action of AOPs. Understanding bacterial adaptation during disinfection plays a vital role in devising strategies to outclass the bacterial survival. Hence, more importance should be given to such studies in the near future for the successful implementation of AOPs.

**Keywords:** Advanced Oxidation Process, Antibacterial resistance, Disinfection, Persistence, ROS, Stress response

## 1. Introduction

The quality of water holds an imperative role in shaping the economy of the world. The consequences of water contaminated with microbial pathogens are often daunting. Around 1.7 billion cases of diarrhoea, mostly due to the gastrointestinal infections caused by the consumption of contaminated water, are reported in children every year, thus resulting in the death of more than 2.2 million people worldwide (“Diarrhoeal disease”, “WHO | Water-related diseases,” 2016). The health aspects of microbial contaminants present in drinking water is well documented in the literature (Ashbolt, 2015; Pandey et al., 2014). The adaptability of microbes, bacteria in particular, against the action of antibiotics at a staggering pace has made the situation more inimical. On the other hand, various biological water treatment methods are now being blamed for facilitating the evolution of antibiotic resistant bacteria (ARB) and the dissemination of antibiotic resistant genes (ARG) (Guo et al., 2017; Zheng et al., 2018). Disinfection strategies are of high asset in treating water because of their performance and efficacy. Among all the disinfection processes, chlorination and UV treatment are well explored for bacterial inactivation and are still practiced worldwide despite their disadvantages like formation of toxic by-products and the possibility of bacterial reactivation (Nyangaresi et al., 2018; Pichel et al., 2019). Moreover, the promotion of ARG holds true for chlorination according to some recent reports (Jia et al., 2019; H. Zhang et al., 2019). Advanced oxidation process (AOP) is another water disinfection strategy which includes processes like ozonation, photocatalysis, photo-Fenton process, electrocatalysis, sonophotocatalysis etc. (Deng and Zhao, 2015).

The commonality in the action of antibiotics and AOPs lies in the production of reactive oxygen species (ROS). While chlorination and UV have direct effects on the DNA, the ROS produced during AOPs target multiple sites in the bacteria viz. membrane, DNA and protein, thereby creating a hostile environment similar to that of antibiotics (Pichel et al., 2019; Van Acker and Coenye, 2017). It is to be noted that in AOPs, ROS is produced in the reactor system containing bacteria and it can further induce oxidative stress inside the cells by forming intracellular ROS, creating an environment akin to that of antibiotics (Giannakis et al., 2016). However, the fate of these effects depends on the bacterial stress response to the pressure caused by the process. This can result in bacterial inactivation, killing or survival. Bacterial stress response to antibiotics is a well-studied subject whereas the studies related to the bacterial reaction during disinfection is mostly limited to the role of commercial

disinfectants and disinfectant by-products in triggering antibacterial resistance (ABR) (Li and Gu, 2019; Poole, 2012; Tkachenko, 2018).

It is the ability of bacteria to attune to the external pressure that enables them to resist the action of antibacterial agents or processes including water disinfection. However, most of the studies on AOP based disinfection focusses on designing the process and synthesis of catalysts, which are indeed important, and neglects the response of bacteria during the process, which ultimately determines the fate of the process on a longer run. In this article, we aim to provide a comprehensive knowledge on the bacterial phenotypic responses and their genotypic determinants that are possible during water disinfection, focussing on AOPs, based on the existing information on bacterial response against stress inducing factors like antibiotics. We expect to bridge this gap by delivering the know-hows of water disinfection which are beyond bacterial inactivation and thus serving few essential strategies which can be considered during the development of water disinfection regiments in the future.

## **2. Stress response and ABR- the extended role of ROS in disinfection**

The history of ABR dates back to early 1940's when bacterial penicillinase was discovered (Abraham and Chain, 1940). Interestingly, this was detected way before penicillin was instilled as a therapeutic agent, pointing at the ability of microbes to survive unfavourable conditions. Decades later, due to the misuse of antibiotics, we are now in an era with the arsenal of active antibiotics diminishing day by day due to the staggering pace of ABR evolution (Alekshun and Levy, 2007). It is of a huge concern especially when biological water treatment plants and some disinfection techniques select ABR and enrich ARGs during the treatment. AOPs are generally not attributed for the selection of ABR, mostly because of the lack of information, and only few data are available in the literature on this aspect. For example, a recent study on the continuous application of ozone resulted in the decrease of the targeted bacteria and ARGs although higher doses of ozone were required to decrease the regrowth potential of the bacteria. (Iakovides et al., 2019). On the contrary, a study conducted on the effect of ozonation on the overall bacterial community and ARGs alluded the selection of bacteria which are GC rich and hence more resistant to the treatment along with the enrichment of few resistant genes (Alexander et al., 2016). Therefore, it can be inferred that the selection of ABR during ozonation depends on the microbial communities present in the water and further enhancement is required to curtail the enrichment of ARGs.

The situation of ARG enrichment and thus water treatment facilities becoming a reservoir for the evolution of drug resistance is very intriguing as we ask what makes a microbe to upregulate its ARGs which are not directly related to the treatment. Now given the fact that majority of bactericidal antibiotics induce ROS formation, especially HO<sup>·</sup>, and it leads to ABR as a result of the oxidative stress response, it is justly possible that similar reaction might be happening inside bacterial cells when they are treated using AOPs (Cirz et al., 2005; Kohanski et al., 2007). As a consequence of bacterial SOS response due to antibiotic induced ROS formation inside the cells, mutation in the DNA occurs via the activation of error-prone polymerases. A comprehensive review on the oxidative stress response associated drug resistance can be found elsewhere (Dwyer et al., 2009). The situation becomes more complex when ROS produced during antibiotic treatment not only helps in resistance acquisition against that antibiotic but also partially protects the bacteria from antibiotics belonging to other classes (Hoeksema et al., 2018). Horizontal gene transfer (HGT) is regarded as one of the key driver of ARG dissemination. It is already known that disinfectants like chlorine and H<sub>2</sub>O<sub>2</sub> at sublethal concentration can promote inter and intra genera HGT (Zhang et al., 2017). Similar experimental analysis on the impact of nanomaterials which are generally used in AOPs indicates a positive correlation with HGT (Li et al., 2020; Lu et al., 2020; S. Zhang et al., 2019). Strikingly, here also ROS plays a major role via the activation of SOS response followed by alteration in the cell membrane permeability and upregulation of the membrane associated HGT genes. Hence, not surprisingly, the bacteria exposed to photocatalysis are also reported to be on the edge of promoting HGT (Guo and Tian, 2019). However, it can be comprehended that all of these convoluted pathways are very unlikely to happen if the production of ROS is harsh enough to foster cell death. AOPs in general work via 3 ways. i) Exogenous production of ROS which attack the bacterial membrane, ii) Diffusion of ROS into the cytoplasm, iii) Endogenous production of ROS by the bacteria itself due to the oxidative stress triggered by exogenous ROS. Indeed, the question of the evolution of ARB will remain relevant only if the ROS produced during AOP is not sufficient enough to kill the bacteria. A graphical representation of ROS induced effects on bacterial survival/death is described in Figure 1.

The use of sublethal concentration of antibiotics has been an essential tool to study the evolution of ARB. Here, a set of bacteria is allowed to survive the treatment either by reducing the antibiotic concentration or the time of exposure and the modifications in their genotypic and phenotypic attributes are analysed to understand how they adapt to the

adversities. This technique was not used to study the intermediate effect of AOPs on the bacterial population until recently. The experimental setup demonstrated by Giannakis *et al* reported the plausible development of ARB evolution in terms of increase in the minimum inhibitory concentration (MIC) upon the exposure of *E. coli* to sublethal solar photo-Fenton reaction (Giannakis *et al.*, 2018). In another study, the perineal exposure of *E. coli* to photocatalysis was found to have an accelerated impact on the ARB evolution (Yin *et al.*, 2020). They further extended their work in understanding the stress response of the bacteria and how ARGs are regulated during the events of photocatalytic exposure. This experimental evidence hints at the role of ROS in eliciting the AR. In contrast, similar studies conducted by our group using sublethal sonophotocatalysis on *Salmonella Typhimurium* resulted in an overall decrease in the MIC of several antibiotics belonging to different class (Rahman *et al.*, 2020). This could be due to the severe pressure offered by the process although similar responses should be verified on other bacterial species before we generalise the effect.

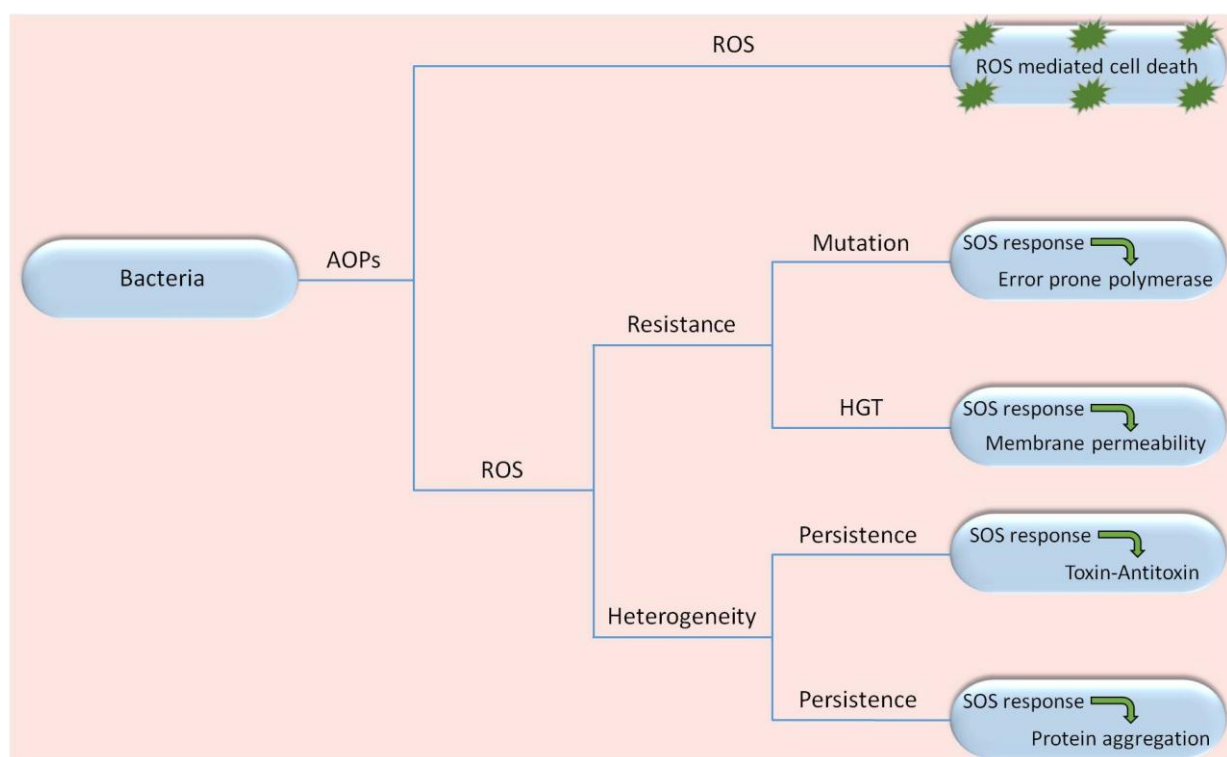


Figure 1. Illustration of the possible effects of ROS produced during AOPs against bacteria

Resistance acquisition is an energy intensive process and is mostly accompanied by a fitness cost. Briefly, the relative growth of ARB in the absence of antibiotics or stressor molecules will be lesser than its isogenic counterpart in order to balance out the expenditure of acquiring resistance (Andersson and Hughes, 2010; Melnyk *et al.*, 2015). While addressing how the oxidative stress rendered by AOPs regulate the fitness cost in ARB, Yin *et al*

cautioned about the accentuating effect of sublethal photocatalysis on reducing the fitness cost of ARB and thereby promoting the proliferation of resistance (Yin et al., 2019). More importantly, if the disinfection process is acting as factor behind compensatory evolution, a phenomenon by which ARB neutralizes the fitness cost, than a mere selective pressure should not be overlooked. Hence, it is very much desirable to use clinically relevant ARB alongside antibiotics for studying the influence of disinfection on the evolutionary traits of bacteria in selecting ARGs.

Disinfection processes were historically designed to kill or inactivate microbes but not to tackle the issue of the emerging ARB and ARGs. Current data also supports the view of bacteria getting resistant to disinfectants like antibiotics (Mc Carlie et al., 2020). The aforementioned data verily opens up the possibility of ROS induced cross resistance to antibiotics in bacteria during AOPs. Signifying the adaptive capability of bacteria against adverse conditions, they indeed pose high risk in causing process resistance unless significant enhancement in the process design and use of proper techniques to validate the cell death after the treatment is not put in place.

### **3. ROS induced heterogeneity in bacteria- a survival strategy**

Heterogeneity is one among the many strategies devised by bacteria while responding to the cues of environmental stress. It can be explained as the tactics deployed by a subset of a clonal population to ward off a particular stress. Interestingly, this state can be achieved either via genetic modifications (genetic heterogeneity) or phenotypic modifications (phenotypic heterogeneity) (Davis, 2020). Phenotypic heterogeneity is a complex trait within an isogenic population in which a subpopulation having special phenotypic features emerge as a result of stress without having much alterations in the genomic content. Bacterial persistence against antibiotics is a good example of phenotypic heterogeneity. Since persistence is often used alongside resistance and tolerance in the scientific literature, a clear distinction in using these terms was proposed recently (Brauner et al., 2016). Briefly, resistance is the ability of bacteria to increase the MIC of an antibiotic, achieved by mutation or HGT, whereas tolerance is defined as the potential to withstand high concentration of antibiotics without changing the MIC. While the feature of tolerance displayed by the whole clonal population is similar to that of resistance, persistence is a non-hereditary form of tolerance exerted only by a subset of bacteria, resulting in a biphasic response to the treatment. Tolerance/persistence can be distinguished using minimum duration for killing

(MDK) assay as it estimates the duration taken by the antibiotic at a concentration exceeding its MIC to kill the bacterial population (Brauner et al., 2016).

Slowing down the metabolic activity and thereby arresting the growth is a key factor which helps in the generation of persisters (Brauner et al., 2016). Among the multiple factors that have been identified as the key regulators of persistence, repression in SOS response appears to be the most important driver of persistence. The SOS response genes which are responsible for the inhibition of cell division were found to have upregulated in the persister population enriched by ampicillin treatment (Gefen and Balaban, 2009). Toxin-antitoxin (TA) modules have also been reported to have important function in bacterial persistence as they are closely associated with bacterial growth arrest (Gerdes and Maisonneuve, 2012). During stress, the antitoxin which inactivates the toxin gets destroyed and eventually the release of toxin results in shutting down the growth promoting factors in bacteria. When MqsR toxin in *E. coli* was made to overexpress, it resulted in the suppression of RpoS mediated stress response and triggered the formation of persisters. Interestingly, the same study indicated the repression of RpoS when the cells were treated with hydrogen peroxide and in turn a spike in persister colonies was observed (Hong et al., 2012). This suggests that the probability of the selection of bacterial persistence in ROS generating environments like AOPs should not be disregarded. This idea was supported by a very recent study where exposing bacteria to several cycles of sublethal photocatalysis improved the bacterial tolerance towards several antibiotics (Yin et al., 2020). Nevertheless, the final outcome of AOPs is likely to be dependent on the level of stress it incurred on the bacteria as understood from the TA modules which promoted persistence and thereby their survival during nominal stress as opposed to the activation of killing pathway when the stress level exceeded the limit of damage repair (Wu et al., 2011)

Bet-hedging is another strategy exercised by a subset of bacteria to evade environmental stress where they preadapt before the onset of a particular stress (Schröter and Dersch, 2019). Although this heterogeneous population carries a significant fitness cost in the environment without having the stress they adapted for, they tend to survive when they are exposed to the stress while the non-adapted get killed in the meantime. This is particularly advantageous for the bacteria which are already exposed to several fluctuating environments. For example, the T3SS (type 3 secretion system) expressing *Salmonella* subset injects effector proteins to the host cells to counteract the lethality of ROS during phagocytosis albeit the heavy fitness cost they carry due to this secretion which results in reduced metabolic activity and thereby



increase in antibiotic tolerance (Diard et al., 2014). Bet-hedging becomes a very important mechanism of bacterial escape from the oxidative stress especially when they are pre-exposed to various scales of oxidative stress. Hence, it is very likely that the reactivated bacterial colonies post disinfection (if any) may show features of heterogeneity. As AOPs are generally considered in the tertiary steps of water treatment, it may have to encounter microbial communities which are pre-adapted to stress. Situation becomes more serious while considering hospital wastewater/ effluents as they foster the diversification of differentially evolved microbes to bypass adversities (Lamba et al., 2017; Petrovich et al., 2020). Hence, we urge the use of clinically and environmentally relevant bacterial strains expressing different phenotypes/genotypes not only for testing the efficacy of disinfection process but also to ensure that it is not selecting characteristics like persistence.

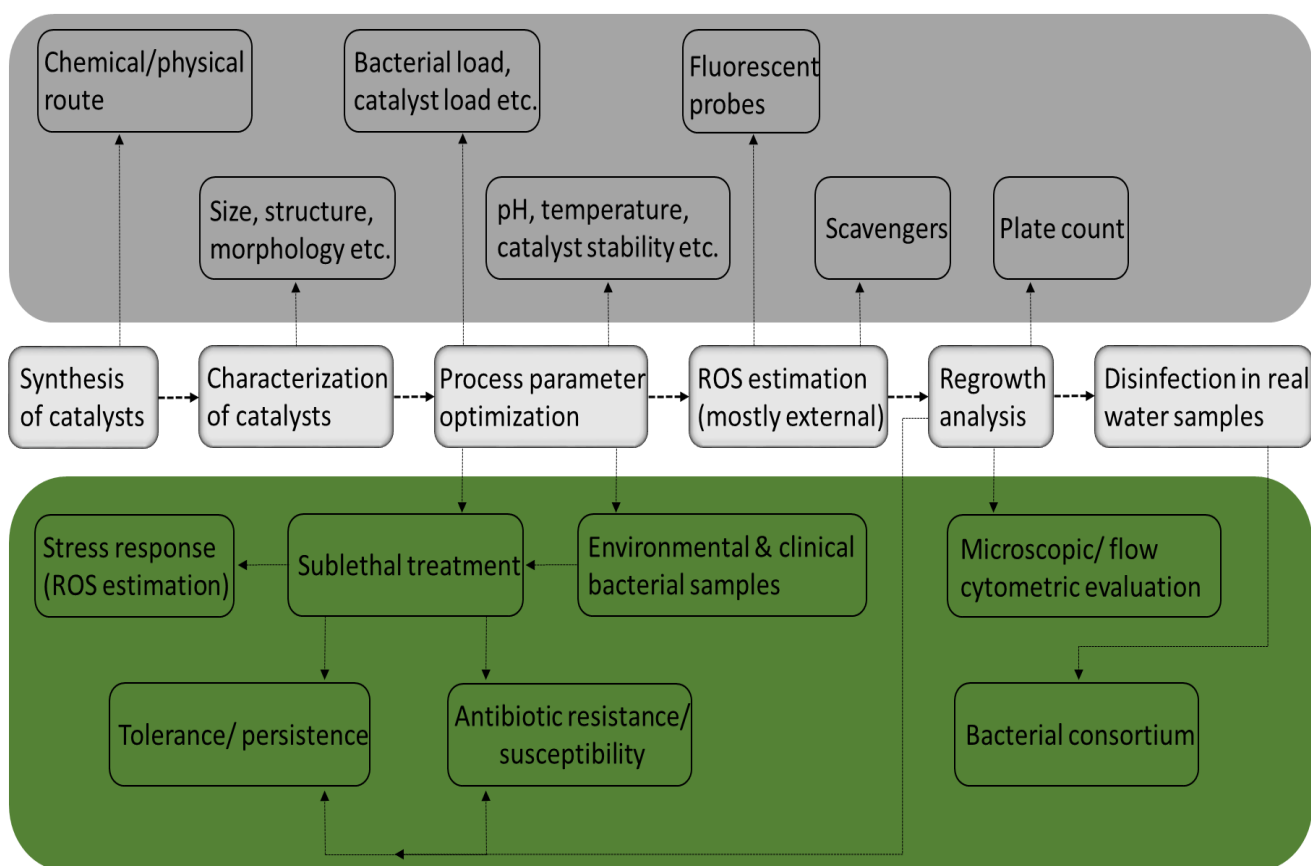


Figure 2. Roadmap describing new strategies (green box) that are proposed to be included during the design of AOP based disinfection modules.

Stress derived protein aggregation in a clonal population also appears to have functions in driving heterogeneity (Mortier et al., 2019). Protein aggregates formed during proteolytic stress are distributed stochastically to one of the cell poles via nucleoid occlusion and the ensuing asymmetric segregation of these proteins during the division of the surviving cells

results in a heterogeneous population (Lindner et al., 2008). In a recent study, Govers *et al* reported that the protein aggregates inherited in *E. coli*, formed as a result of sublethal heat shock, elevated the heat resistance compared to its isogenic counterpart devoid of protein aggregates (Govers et al., 2018). Interestingly, it also improved the resistance against the proteolytic stress inflicted by ROS. A report by Shi *et al* proposed protein aggregation as the reason behind the bactericidal activity of photocatalysis using silver nanoparticles (AgNPs), refuting the existing evidences on the ROS generation and its associated lethality (Shi et al., 2019). They suggested the possibility of AgNPs acting as a mediator to transfer the light energy to the proteins, resulting in protein aggregation followed by cell death. Based on the proteomics data, they also claimed the damage to be irreversible and therefore it is very unlikely to have bacterial resistance against AgNPs in the presence of light. However, to validate the protein aggregation mediated cell death and to rule out the possibility of persistence associated, microscopic tools and MDK assay should have been performed respectively especially on the grounds of reports on the bacterial resistance to AgNPs and persistence linked with protein aggregation (Govers et al., 2018; Panáček et al., 2018). Keeping the complexities associated with bacterial adaptation in mind, studies related to bacterial stress response during the disinfection would help to tweak the system in order not to get selected for traits like persistence.

#### 4. Conclusions

The common feature shared by most of the antibiotics and AOP based disinfection processes is the production of ROS. The fate of bacterial survival under the action of ROS depends on the magnitude of the ROS production. Bacteria have evolved different strategies to outmanoeuvre the stress caused during antibiotic treatment. Like so, it is justly possible that they can bypass the lethal actions of AOPs unless effective measures are taken to curtail the stress response. Based on the existing knowledge on the bacterial stress response against antibiotics, we propose to extend this information for the betterment of AOP based disinfection (Figure 2) as follows

- Inclusion of sublethal AOPs to study the bacterial response and adaptation to the process via the plausible development of resistance and persistence to different antibiotics.
- Use of clinically and environmentally adapted bacteria for the disinfection process along with their wildtypes.

- Exploration of disinfection efficacy against bacterial consortia in their natural form.
- Use of microscopic and flow cytometry based tools to validate the bacterial death.
- Above all, increase the process efficiency by increasing the ROS production, for example: development of nano-catalysts with superior ROS producing capacity.

### **Conflicts of interest**

There are no conflicts to declare.

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