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Assessment of the disinfection capacity and eco-toxicological impact of atmospheric cold plasma for treatment of food industry effluents

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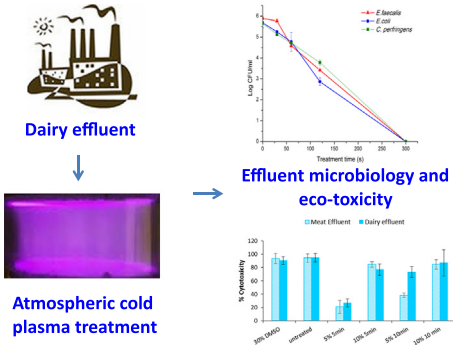
HIGHLIGHTS

- ACP is an effective alternative solution for wastewater treatment.
- Effective to inactivate key mono/mixed indicator bacteria from model effluents
- ACP shows useful efficacy within short periods of both treatment and retention times.
- System and treatment parameters affect the bacterial inactivation efficiency.
- Treated samples displayed limited effect on test species up to 24 h exposure, a prolonged contact of up to 48 h was toxic.

GRAPHICAL ABSTRACT



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ABSTRACT

Generation of wastewater is one of the main environmental sustainability issues across food sector industries. The constituents of food process effluents are often complex and require high energy and processing for regulatory compliance. Wastewater streams are the subject of microbiological and chemical criteria, and can have a significant eco-toxicological impact on the aquatic life. Thus, innovative treatment approaches are required to mitigate environmental impact in an energy efficient manner. Here, dielectric barrier discharge atmospheric cold plasma (ACP) was evaluated for control of key microbial indicators encountered in food industry effluent. This study also investigated the eco-toxicological impact of cold plasma treatment of the effluents using a range of aquatic bioassays. Continuous ACP treatment was applied to synthetic dairy and meat effluents. Microbial inactivation showed treatment time dependence with significant reduction in microbial populations within 120 s, and to undetectable levels after 300 s. Post treatment retention time emerged as critical control parameter which promoted ACP bacterial inactivation efficiency. Moreover, ACP treatment for 20 min achieved significant reduction (≥2 Log_{10}) in *Bacillus megaterium* endospores in wastewater effluent. Acute aquatic toxicity was assessed using two fish cell lines (PLHC-1 and RTG-2) and a crustacean model (*Daphnia magna*). Untreated effluents were toxic to the aquatic models, however, plasma treatment limited the toxic effects. Differing sensitivities were observed to ACP treated effluents across the different test bio-assays in the following order: PLHC-1 > RTG-2 ≥ *D. magna*; with greater sensitivity retained to plasma treated meat effluent than dairy effluent. The toxic effects were dependent on concentration and treatment time of the ACP treated effluent; with 30% cytotoxicity in *D. magna* and fish cells observed after 24 h of exposure to ACP treated effluent for concentrations up to 5%. The findings suggest the need to employ wider variety of aquatic organisms for better understanding and complete toxicity evaluation of long-term effects.

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The study demonstrates the potential to tailor ACP system parameters to control pertinent microbial targets (mono/poly-microbial, vegetative or spore form) found in complex and nutritious wastewater effluents whilst maintaining a safe eco-toxicity profile for aquatic species.

1. Introduction

Food processing industries are one of the largest sources of wastewaters, with a trend of increasing volumes being produced. Water plays a vital role in the food industry with large volumes of wastewater derived from various processing units including washing, cleaning, sanitization and various steps in the manufacturing of food products themselves. The physical and chemical properties of the effluents derived from the food sector vary in line with the product type and quantity. Therefore, the wastewater streams can hold a multiplicity of microbiological and chemical contaminants within an environment characterized by high amounts of organic content, nutrients like proteins, carbohydrates, fats, minerals and higher concentrations of suspended solids; biological oxygen demand (BOD) and chemical oxygen demand (COD), with large variation in pH (Gough et al., 2000; Perle et al., 1995).

Treatment methods currently applied to wastewaters include electrochemical treatment, anaerobic processing, ultrafiltration, chlorination, heat treatment, radiation treatment and different combinations of these (Demirel et al., 2005; Yavuz et al., 2011). The increasing costs involved for treatment methods, residual chemical by products, high energy consumption along with the stringent standards for wastewater treatment, comprise some of the major limitations of these technologies. Public health authorities and environment management systems explore innovative methods to mitigate the environmental impact of the wastewaters discharged from these organic intensive industries. Atmospheric cold plasma is under intensive investigation as a novel decontamination technology. Plasma comprises of charged particles (positive and negative electrons), free radicals, UV photons and wide range of reactive species such as hydrogen peroxides, nitric oxide derived species. Non-thermal plasma or cold plasma generated from atmospheric or near atmospheric pressure at room temperature is called atmospheric cold plasma. Cold plasma is characterized by non-equilibrium, where cooling of the ions and the uncharged molecules is significantly more effective than that of energy transfer from electrons resulting in the gas remaining at a low temperature (Bourke et al., 2017). The introduction of an electric discharge into the liquid surface causes a number of chemical reactions (Fridman, 2008; Locke et al., 2012). The oxidative degradation of the organic matter and biological content is effected through the ensuing reactive species and the effects of the secondary reactive species are longer lived and mediated through liquids. The basis of the treatment is transfer of the charged species at the gas-liquid interface (Pivovarov and Tischenko, 2005). Non-thermal plasma technology has been studied to degrade phenol pollutant dyes (Sarangapani et al., 2017a) and for reducing COD and TOC (Reddy et al., 2013; Tomizawa and Tezuka, 2007) in effluents. Also, ACP has proven effective for the wide range of pathogenic and spoilage microorganisms predominantly found in food and biomedical sectors. Studies from Han et al. (2014) demonstrated effective bacterial inactivation using in-package DBD-ACP system within short period time and post treatment storage time of 24 h. Also work by Pavlovich et al. (2013) demonstrated effective decontamination of bacteria with 120 s of treatment present in phosphate buffer using indirect dielectric barrier discharge treatment ACP system. Although ACP studies have been focused on aqueous treatment, there are limited studies pertaining to its application in microbial contamination in complex media compositions like in wastewater effluents with much shorter contact time.

Several studies report significant microbial concentrations and the presence of pathogens in the food industrial environment (McGarvey et al., 2004; Parkar et al., 2015). A wide range of microbial profiles have been reported in food effluents including *Cryptosporidium parvum, Giardia, Escherichia coli, Clostridium perfringens, Enterococcus faecalis*, *Salmonella* etc. (Chapman, 2000; Dungan and Leytem, 2013; Ibekwe et al., 2003). Since food industrial wastes contain high levels of organic content, they act as an ideal medium for the growth and survival of both pathogenic and spoilage bacteria within the industrial environment, causing serious challenges for the efficient removal of organics and the safety profile of the wastewater effluent. The effluents released from the food industries are characterized by high biological oxygen demand (BOD) and chemical oxygen demand (COD). The bacteria decompose the organic materials present in the wastewater, depleting the oxygen levels in rivers proving lethal for most aquatic life (Enderlein et al., 1997).

This study focussed on two sectors and employed meat and dairy model effluents to address several outstanding research objectives. These were to ascertain the impact of ACP on bacterial communities prevalent in wastewaters and to devise parameters for their efficient control using custom built DBD-ACP system. Target bacteria were selected based on common indicator organisms encountered in the food industries and as recommended by EPA (USEPA, 2010). Considering the most predominant and resistant of pathogens, the principle indicator microorganisms *Escherichia coli, Clostridium perfringens Enterococcus faecalis* and *Bacillus megaterium* endospores were selected. A better understanding of microbial inactivation mechanism by ACP and the behaviour of multi-species communities will promote the definition of treatments that either alone or in combination with other control agents will assure the effective decontamination of food derived wastewater streams.

Additionally, considering that the longer term effects of ACP on biological systems are mediated through liquid, it was investigated if this technology can be safely deployed and what the impact of ACP treated effluent on eco-toxicity markers may be. The aquatic markers selected for eco-toxicity testing were fish cell lines (PLHC-1 and RTG-2 cells) and crustaceans (*Daphnia magna*) representative of a range of trophic levels. These are simple test organisms which are known to be sensitive to a wide range of pollutants and have a standard reproducible response to facilitate comparison.

2. Material and methods

2.1. Preparation of model effluent

Model dairy effluent (DE) of pH 6.0 was prepared as reported by Daverey and Pakshirajan (2011) which contained 2 g/l semi-skimmed milk powder (TESCO, Ireland), 0.2% (w/v) milk fat (East end Foods, UK), 0.01% (w/v) sodium hydroxide (Sigma-Aldrich, Ireland) and sterile distilled water. The milk powder was composed of 35% proteins, 50.3% carbohydrates, 0.6% fat, 1.05% calcium, 0.8% phosphorus, 0.095% magnesium. The organic content in dairy effluent varies with the manufactured product; therefore, the relative impact of organic milk fat content on bacterial inactivation efficiency was examined. The milk fat content as an experimental variable ranged between 0.2 g/l to 4 g/l.

Model meat effluent was prepared using procedure of Barrera et al., with few modifications. The synthetic meat effluent consisted of commercial meat extract powder (Scharlau Chemie, Barcelona, Spain) 1950 mg/l, glycerol ($C_3H_8O_3$) (Sigma-Aldrich, Ireland) 200 mg/l, ammonium chloride (NH_4Cl) (Sigma-Aldrich, Ireland) 360 mg/l, sodium

chloride (NaCl) 50 mg/l in sterile distilled water. The effluents were prepared as required and the concentration was maintained throughout the study.

2.2. Bacterial strains and growth conditions

Bacterial strains of *Escherichia coli* NCTC 12900 (non-toxigenic O157:H7), *Enterococcus faecalis* ATCC 29212 and *Bacillus megaterium* LZB026 were obtained from the bacterial culture collection of Dublin Institute of Technology while *Clostridium perfringens* ATCC 13124 was obtained from Microbiologics®, Ireland. Stock cultures were kept frozen at −80 °C in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK). The working culture of *E. coli* and *B. megaterium* was prepared on tryptone soy agar (TSA, Biokar Diagnostics, Ireland), *E. faecalis* on brain heart infusion agar (BHIA, Biokar Diagnostics, Ireland) and *C. perfringens* on reinforced clostridial agar (RCA, Biokar Diagnostics, Ireland), respectively. All the culture plates were incubated aerobically except *C. perfringens* which was incubated anaerobically using Anaerocult A (Merck, GasPak®) at 30–37 °C for 24 h and later stored at 4 °C.

2.3. Preparation of bacterial suspensions

A subculture of each strain was obtained by inoculating *E. coli* in tryptic soy broth (TSB, Scharlau Chemie, Barcelona, Spain), *E. faecalis* in brain heart infusion broth (Sigma-Aldrich, Ireland) and *C. perfringens* in thioglycollate broth (Sigma-Aldrich, Ireland) and incubating it at 30–37 °C for 24 h. The cells were harvested by centrifugation at 13,000 rpm for 4 min. The supernatant was removed and washed twice with sterile phosphate buffer solution (PBS, Sigma-Aldrich, Ireland). The cell pellet was re-suspended in PBS. The bacterial density was determined using a 600 nm spectrophotometer and confirmed with plate count. The final bacterial concentration was adjusted to 10^{5-6} CFU ml^{-1} in the model effluents.

2.4. Microbiological enumeration

For microbial enumeration, the samples were taken before and after plasma treatment respectively. Control and treated samples were serially diluted in maximum recovery diluent (MRD, Merck) and spread plated onto appropriate media, i.e. TSA for *E. coli*, BHIA for *E. faecalis* and RCA for *C. perfringens*. Surviving bacterial count was determined after 24 h of incubation. All experiments were performed in triplicate. The bacterial inactivation levels were determined by plotting Log_{10} CFU ml^{-1} of survival bacteria against the treatment time (s) for each experimental organism. In order to obtain low microbial detection limits, 1 ml and 0.1 ml of the treated sample (undiluted) was spread plated on appropriate media. The limit of detection was Log_{10} 1.0 CFU ml^{-1}.

2.5. Preparation and treatment of mixed bacterial culture in effluents

A cocktail of microorganisms was used to represent the probability of a variable microbial load in dairy and meat wastewater. Three different bacteria were used including (1) *E. coli*, (2) *E. faecalis*, and (3) *C. perfringens*. The overnight cultures were purified and washed with PBS as described previously. Equal volumes of each bacterial culture were mixed together to obtain a final concentration of approx. 10^5 CFU ml^{-1} and were added to the model effluent. The mixed bacterial culture samples were then exposed to ACP at 80 kV for different treatment times of 1 to 10 min, respectively. The surviving bacterial population and concentration were determined by surface plating on appropriate selective medium for each bacterium; cefixime-tellurite sorbitol MacConkey (CT-SMAC, Biokar diagnostics, Ireland) agar for *E. coli O157:H7*, modified Membrane enterococcus Slanetz and Bartley agar (mEI agar, Oxoid, Ireland) for *E. faecalis* and modified Tryptose sulfite cycloserine Agar (mTSC) with D-cycloserine supplement (Biokar diagnostics, Ireland) for *C. perfringens*. All plates were incubated at 37 °C for 24 h. The plates were incubated for additional 24 h to observe any subsequent increase in visible colonies.

2.6. Preparation and analysis of B. megaterium spores

Spores of *B. megaterium* were cultivated as per the method described by Garvey et al. (2013) with modification. Spores were prepared by growing the bacterial strain on TSA supplemented with 3 mg/l of manganese sulphate for 8 days at 30 °C. Spores were collected by flooding the plate with 10 ml of PBS and rubbing the surface gently with a sterile spreader. After harvesting, the cells were washed twice with PBS by centrifugation at 10,000 rpm for 10 min at 4 °C. The samples were then heated at 85 °C for 25 min to inactivate any vegetative cells and immediately cooling it in ice water bath. After heating, spores were washed twice by centrifugation (10,000 rpm for 10 min at 4 °C) in sterile PBS. The purity of the sample was determined using malachite green spore staining method and phase contrast spectroscopy (Rowan et al., 2001). The concentration of the spore suspension was determined by plating onto TSA agar plates and incubating aerobically at 30 °C for 24 h. The spore suspension prepared was stored at 4 °C until use.

2.7. Atmospheric plasma treatment setup and treatment

The plasma treatment was performed using dielectric barrier discharge (DBD) ACP system (DIT-120) which is an in-house set up at Dublin Institute of Technology with a maximum voltage output of 120 kV at 50 Hz and has been described in Han et al. (2015) and Sarangapani et al. (2017a). The distance between the two electrodes was maintained at 30 mm for liquid studies. Aliquots (10 ml) of wastewater samples were subjected to different doses of direct plasma treatment. System and process parameters included voltage level (60–80 kV), treatment time (0–30 min), retention time (0–10 min), post treatment storage time (0–24 h) and organic fat content (0.2–4 g/l). All treatments were performed at ambient temperature (16 °C to 18 °C) using atmospheric air as the working gas. The temperature increase inside the container after plasma treatment observed was <5 °C.

2.8. Determination of reactive species concentration after ACP treatment

Hydrogen peroxide concentration was determined by oxidation of potassium iodide (KI) to iodine and spectrophotometrically measured at 405 nm. Briefly, 50 μl of plasma treated dairy effluent/standard curve samples, 50 μl of phosphate buffer and 100 μl of KI was added to 96 well microtiter plate. After 20 min incubation at room temperature absorbance was measured at 405 nm. A standard curve with known hydrogen peroxidase was included on the same plate to convert absorbance into peroxide concentration.

Nitrite and nitrate concentration was measured using Griess reagent (Sigma-Aldrich, Arklow, Ireland) and Spectroquant® nitrate spectrophotometric assay kit (Merck Chemicals, Darmstadt, Germany) as described in Lu et al. (2017).

2.9. Cell culture and cytotoxicity test

RTG-2 cells (Catalog No. 90102529) derived from rainbow trout gonad and PLHEC-1 (CRL-2406) derived from a hepatocellular carcinoma in the topminnow were obtained from FOCAS research Institute (Dublin, Ireland). Both the cells were maintained in Dulbecco's modified medium nutrient mixture/F-12 Ham (DMEM) supplemented with 10% (RTG-2) or 5% (PLHC-1) fetal bovine serum (FBS) and 45 IU ml^{-1} penicillin, 45 mg ml^{-1} streptomycin, 25 mM HEPES and 1% non-essential amino acids. Cultures were maintained in a refrigerated incubator at 20 °C (RTG-2) or 30 °C (PLHC-1) under normoxic atmosphere.

For cytotoxicity test, individual wells of 96-well microplate were seeded with 100 μl of cell suspension at seeding density of 2×10^5 cells per ml of RTG-2 cells and 1×10^5 cells per ml of PLHC-1 cells and

were allowed to attach for 24 h exposure period. ACP treated effluent concentrations (5, 10, 20%) were prepared in respective supplemented DMEM media. After 24 h of cell attachment, the cells were washed with PBS and exposed to different concentration of test ACP concentrations. Six replicate wells were used for each controls and ACP test concentrations per microplate. Cytotoxicity test was assessed using Alamar blue (AB) and Neutral red (NR) assay which was conducted on the same plate following the methodology as previously described by Davoren and Fogarty (2006).

2.10. Eco-toxicological analysis using Daphnia magna acute immobilization test

Acute toxicity tests were performed with the ACP treated effluents in accordance with the OECD test 202 Guidelines. *Daphnia magna* was kindly supplied by FOCAS research Institute cultured in static conditions at 21 °C and under a 16 h/8 h light/dark photoperiod for all exposures. Toxicity tests were performed on *D. magna* neonates that were <24 h old. Concentrations of ACP (5 and 10 min) treated effluents was set to 2, 5, 10, 20% using Elendt M4 Daphnia medium. Three replicates were tested for each test and control samples and 5 neonates were used for each replicate. Immobilization of neonates was determined visually after 24 h and 48 h of exposure; any abnormalities or signs of stress were also recorded. The results obtained were used to calculate EC_{50} values i.e. effective concentration at which 50% of exposed neonates die.

2.11. Statistical analysis

Each experiment was performed in triplicate (three independent exposures) to ensure reproducibility. The experimental data was analysed by two-way ANOVA using IBM SSPS 22.0 (SPSS Inc., Chicago, USA). For the inactivation kinetics studies between bacterial strains, two variance analyses were performed using *t*-test variance test.

For all cell assay (AB/NR assay) fluorescence as fluorescence unit (FUs) was measured using microplate reader. Cytotoxicity was expressed as mean percentage inhibition relative to unexposed control values, which was calculated using formula [100 − (mean experimental data / mean control data × 100)]. The cytotoxicity of the control sample was set to 0%. Statistical analysis and curve fitting were performed to calculate the EC_{50} values using a nonlinear-regression sigmoidal dose-response curve model provided by Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The EC_{50} values are reported ±95% Confidence Intervals.

3. Results and discussion

A sequence of experiments was performed to determine the best treatment parameters for bacterial inactivation efficiency within complex effluents using laboratory scale DBD-ACP system. Contained post treatment storage time ranging from 1 to 24 h was previously reported as an effective tool to enhance the microbial inactivation efficacy of ACP (Han et al., 2014; Ziuzina et al., 2014). Additionally, we have reported that the biological activity associated with plasma reactive species is retained in liquids in a more stable form (Han et al., 2016b; Ziuzina et al., 2013), warranting an evaluation of post treatment retention time effects in treated liquid effluents. Therefore, with respect to continuous or batch waste water decontamination treatment, the retention time prior to analysis can be considered in tandem with treatment time and other variables in order to optimize the processing treatment regimen. Thus, this study evaluated a post treatment retention time in open conditions, treatment time, voltage level, effluent composition and the nature of the microbial challenge in that order.

3.1. Effect of retention time on bacterial inactivation

Post treatment retention time (PTRT) studies were performed in simple phosphate buffer solution in the presence of *E. coli* and *E. faecalis*. Post treatment retention was carried out by holding the opened wastewater sample post ACP treatment. Fig. 1 shows the inactivation of *E. coli* after ACP treatment at 80 kV after a fixed 1 min of plasma treatment time followed by variable PTRT of 0, 5 or 10 min. *E. coli* and *E. faecalis* populations decreased with increasing retention time and no cells were detected after 10 min of PTRT. When the plasma treatment time was reduced to 30 s, the same trend was repeated where microbial loads went from 5 Log_{10} to 1 Log_{10} CFU ml^{-1} after 10 min of retention time (data not shown). Similarly, *E. faecalis* was undetectable after 1 min of ACP treatment with 10 min of retention time. Thus, retention time emerged as a critical processing parameter for microbial control and a retention time of 10 min after 1-min treatment at 80 kV of voltage was sufficient for effective bacterial inactivation. These results indicate the high efficiency of ACP at short treatment and retention times which can assist in fast processing rates of wastewater decontamination industrially.

3.2. Effect of voltage level and treatment time on bacterial inactivation efficiency

The relationship between treatment time and applied voltage level is shown in Table 1. Increasing voltage level gradually enhanced the rate of microbial inactivation in DE, which was most notable after 2 and 5 min of ACP treatment for all bacteria examined. Bacterial inactivation at 80 kV in relation to ACP treatment time in dairy and meat effluent is further summarized in Fig. 2**.** The initial bacterial concentration inoculated in model effluent samples before treatment was 5.6 ± 0.1 Log_{10} CFU ml^{-1} of *E. coli,* 5.9 ± 0.3 Log_{10} CFU ml^{-1} of *E. faecalis* and 5.3 ± 0.3 Log_{10} CFU ml^{-1} of *C. perfringens* respectively. Significant reduction of ±1 Log_{10} unit of bacterial concentration was observed after 1 min of the ACP treatment ($p < 0.05$) in the dairy effluent. However, bacterial indicators in meat effluent were found to be much more sensitive to plasma treatment, with most bacterial populations being affected at 1 min of ACP treatment and showing complete inactivation after 2 min of treatment. Therefore, increasing the ACP treatment time with a PTRT of 10 min significantly enhanced the rate of bacteria inactivation,

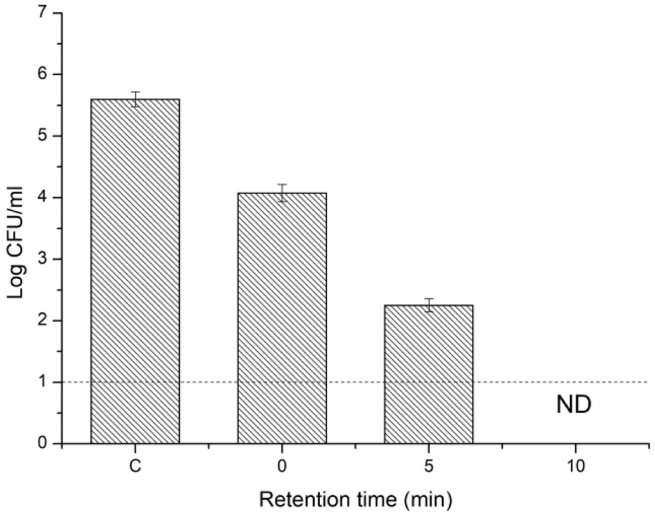

Fig. 1. Effect of post treatment retention time (0, 5, 10 min) on *E. coli* treated with DBD ACP system for 60s at 80 kV in PBS. Dotted line indicates limit of detection (1 log_{10} CFU ml^{-1}). Vertical bars represent standard deviation. ND: Non-detectable.

Table 1
Effect of Atmospheric cold plasma on bacteria inactivation at various applied voltage levels with 10 min PTRT.

Voltage level (kV)	Treatment time (min)	*E. coli*		*E. faecalis*		*C. perfringens*	
		Log_{10} CFU/ml	SD[a]	Log_{10} CFU/ml	SD[a]	Log_{10} CFU/ml	SD[a]
60	0	5.68	0.23	5.68	0.16	5.65	0.22
	1	4.92	0.11	4.46	0.003	4.77	0.23
	2	4.79	0.07	4.14	0.08	4.42	0.15
	5	ND	–	2.67	0.05	ND	–
70	0	5.62	0.09	5.70	0.03	5.65	0.22
	1	4.61	0.02	4.87	0.12	4.70	0.09
	2	3.95	0.19	4.34	0.10	3.55	0.21
	5	ND	–	ND	–	ND	–
80	0	5.68	0.23	5.90	0.08	5.32	0.11
	1	4.77	0.08	4.58	0.03	4.49	0.07
	2	2.87	0.18	3.42	0.03	3.78	0.13
	5	ND	–	ND	–	ND	–

[a] SD = standard deviation, ND = Non-detectable.

with no culturable cells detected after 5 min treatment. The limit of detection for bacterial recovery was 1.0 Log_{10} CFU ml^{-1}.

ACP treatment generates several reactive species which are excellent sources of ROS and RNS. These reactive species are mediated in secondary stable forms in liquids and penetrate into bacterial cells and membranes causing damage to membranes or intracellular functions (Han et al., 2016a). Erosion of cell components affects the cell membrane causing oxidation of free fatty acids, lipids and protein components. This etching activity leads to irreversible damage to DNA, RNA and enzymes eventually causing cell lysis or cell death (Daş et al., 2006; Dobrynin et al., 2009). The highest applied voltage and the longest treatment time yielded the highest rate of inactivation. The high voltage levels in addition to longer treatment times in a contained environment may also contribute to increased concentration of active species amplifying the adverse effect on nucleic acids, affecting DNA with the loss of cell culturability. This is in agreement with earlier studies (Han et al., 2014; Janex et al., 2000; Liu et al., 2013) which demonstrated the relationship between the reactive species and higher voltage and treatment time that influence the bacterial inactivation rate by reducing bacterial metabolism or by changing the permeability of the cell membrane.

3.3. Effect of the organic fat content on bacterial inactivation with ACP treatment

The relative amounts of the fats and proteins in wastewaters from the dairy or meat industry can vary considerably and may not be of routine concentration or composition, even within a sector. A rich organic environment can impact on the antimicrobial efficacy of ACP (Han et al., 2016b). The active species generated not only react with the target contaminant but also with degraded products of organic components within the effluent. The bacterial inactivation efficiency of ACP was further challenged with two synthetic wastewater samples containing additional fat content, at 4 g/l and 2 g/l by comparison with the initial dairy effluent composition (0.2 g/l milk fat). The bacterial inactivation efficiency by ACP in fat rich effluent was slower than in the lower fat content effluent. The protective effect of organic matter upon the bacterial cells during ACP treatment and the variability in levels that may challenge a process must be considered (Fig. 3).

The organic matter present in the dairy effluent can scavenge reactive species preventing their interaction with the bacterial cells present. The reactive species react and oxidize the organic content present in the dairy effluent, in particular double bonds of unsaturated compounds to low molecular compounds (El-Sayed et al., 2015) thus making them unavailable to interact with the bacteria. However, as the treatment time increased to 5 min the ACP can effectively inactivate the target microorganisms in dairy wastewater; the fat content induced shoulder on inactivation can be overcome by extending the treatment time. Similar patterns of treatment environment related resistance to ACP generated reactive species were obtained by Ziuzina et al. (2015) and Han et al. (2016b) who evaluated the Reactive Oxygen Species (ROS) concentration generated post ACP treatment of bacterial cells suspended in various liquid media. Distinct differences in bacterial inactivation efficiencies were observed across media, with lower levels of ROS observed in beef extract and lettuce broth by comparison with PBS, although the same ACP treatment parameters were used for all the suspensions. The low ROS concentration detected may reflect a scavenging effect of the media, allowing bacterial cells to recover and grow.

Further chemical characterization of plasma treated dairy and beef lipids performed by Sarangapani et al. (2017b) gives insights on cold plasma-food interactions in terms of quality effects, particularly for oils and fats present in food industry effluent. The plasma process was found to oxidize the fat content into simpler fatty acids study along

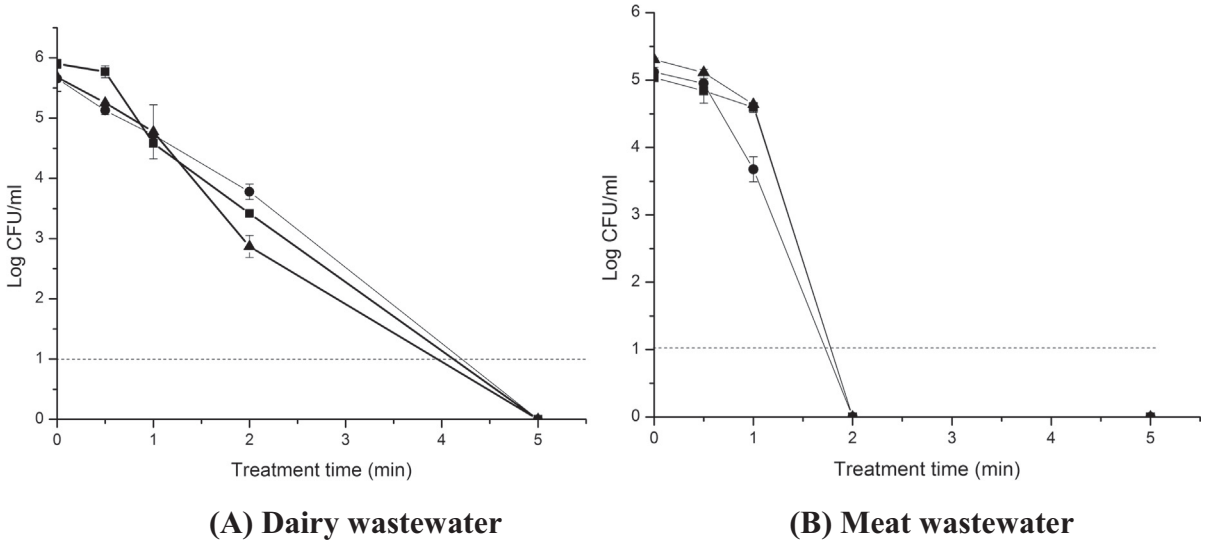

Fig. 2. Effect of ACP treatment time on (▲) *E. coli* (■) *E. faecalis* and (●) *C. perfringens* in (A) dairy (B) meat wastewater treated at 80 kV with post treatment retention time of 10 min. Dotted line indicates limit of detection (1 log_{10} CFU ml^{-1}). Vertical bars represent standard deviation.

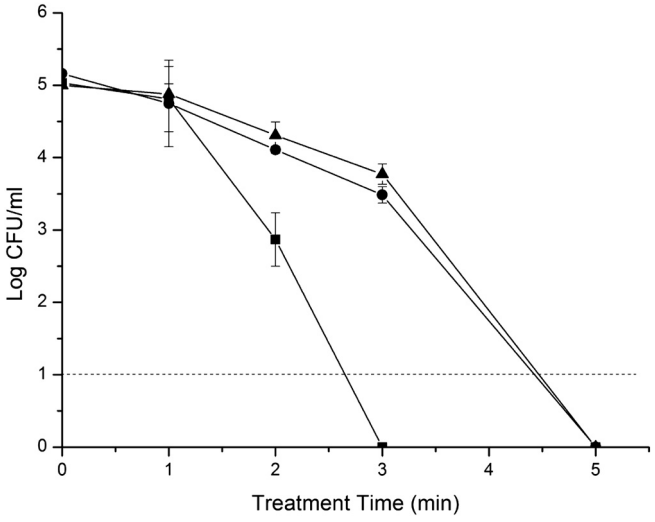

Fig. 3. Inactivation of *E. coli* in dairy effluent at different fat content (■) 0.2 g/l (●) 2 g/l (▲) 4 g/l. Dotted line indicates limit of detection (1.0 log10 CFU ml^{-1}). Vertical bars represent standard deviation.

with formation of oxidation products 2-nonenal, azelaic acid, 9-oxononanoic acid, nonanoic acid and octanoic acid. However, these oxidation products were identified only after extended treatment time of 30 min.

Findings from the current study illustrate the efficacy of ACP depends on water matrix/organic content as well as treatment time. Food industrial effluents vary in nutrient/organic content and concentrations. The final inactivation efficacy may therefore be influenced by further factors such as product parameters including potential to scavenge reactive species as well as significant dependency on the ACP system prototype geometry and treatment conditions.

3.4. Inactivation of mixed culture bacteria from wastewater effluent

In order, to evaluate the performance of ACP treatment for poly microbial challenges a bacterial cocktail was prepared in DE. A mixed culture prepared using *E. coli, E. faecalis* and *C. perfringens* was treated with ACP at 80 kV for different treatment time periods (Fig. 4). Significant

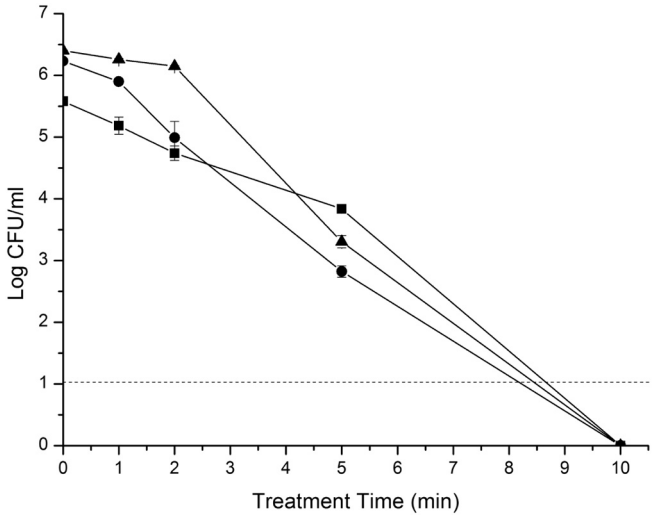

Fig. 4. Inactivation of mixed culture bacteria in dairy wastewater effluent treated with ACP at 80 kV. Dotted line indicates detection limit of 1.0 Log_{10} CFU/ml. Vertical bars represent standard deviation. (■) *C. perfringens* (●) *E. coli* (▲) *E. faecalis.*

reduction ($p < 0.05$) in the mixed culture was observed after 5 min of ACP treatment, showing rapid reduction of *E. coli* and *E. faecalis* to levels of 3.0 to 3.4 Log_{10} CFU ml^{-1}. *C. perfringens* appeared to be significantly more resistant to ACP treatment ($p \leq 0.05$), showing reduction of 1.7 Log_{10} CFU ml^{-1} after 5 min of ACP treatment. Complete elimination of mixed culture bacteria required a doubling of treatment time to 10 mins. However, prolonging the treatment time to 10 min reduced all bacterial population below detection limits. The inactivation rate of the mixed culture cocktail was slower by comparison to the respective mono culture preparations, which may be due to the overall higher bacterial load and the greater potential for organic based quenching of the reactive species generated by ACP treatment which are ultimately responsible for bacterial cell death. Different membrane structures could lead to different relative resistance between the bacterial strains (Han et al., 2015), however, further fundamental insights are required into how poly-microbial communities behave in response to the stresses imposed by ACP.

3.5. Generation of reactive species

The atmospheric plasma discharge is an effective source of reactive oxygen and nitrogen species (RONS). Hydrogen peroxide (H_2O_2), nitrates (NO_3^-) and nitrites (NO_2^-), are biologically active species known to induce cell proliferation and cause substantial degrees of damage to bacterial cells. In the current study, concentrations of H_2O_2, NO_2^- and NO_3^- for different organic fat content DE after ACP exposure was quantitatively measured. Hydrogen peroxide levels in treated dairy effluent was in the range of 38–500 μM H_2O_2 depending on treatment duration and milk fat content present in the dairy effluent. Within 5 min of plasma treatment, significantly higher concentration of 500 μM of H_2O_2 was detected in 0.2 g/l milk fat content DE samples (Fig. 5A). These finding correlated to the bacterial inactivation studies, where increasing plasma exposure resulted in increased bacterial inactivation and showed linear correlation to the concentration of H_2O_2 generated in the solution. Very low concentrations of H_2O_2 were available in 2 g/l and 4 g/l milk fat dairy effluent for contact with the bacterial cells, reflecting the ability of this complex lipid rich solution to scavenge large amounts of H_2O_2.

The concentration of nitrate is presented in Fig. 5B, which was higher in 4 g/l and 2 g/l milk fat content DE samples and increased after ACP exposure: for treatment time of 3 min the concentration of NO_3^- reached values of 715 μM and 627 μM. The complexity of initial DE samples, consisting of high proteins, fats, lipids contributes to overall nitrogen content leading to higher nitrate concentration after plasma treatment.

Reactive species in this study were recorded immediately after ACP treatment (including post treatment retention time). Both plasma reactive species generated; hydrogen peroxide and nitrates are long lived species which showed treatment time dependence, which could improve the microbicidal effect. Previous studies from this DBD-ACP system, demonstrated stable concentration of H_2O_2 in PBS for several weeks (Boehm et al., 2016). Further work from Julák et al. (2012) showed persistent microbicidal effect in water exposed to the corona discharge during 4 weeks of storage. Although ozone (O_3) completely and H_2O_2 almost disappears, the plasma treated water remains antimicrobial for one week or more following its initial exposure to plasma. Our studies showed increasing concentration of generation of hydrogen peroxide and nitrates however, further studies are required for long terms of stability of ACP in nutrient rich treatment samples like food industry effluents.

3.6. Inactivation of B. megaterium spores in dairy and meat effluent

B. megaterium spores were used as a working alternative and challenge model in lieu of *Cryptosporidium* oocysts (Garvey et al., 2013). The spores were suspended in wastewater effluents and subjected to continuous ACP treatment. Significant difference ($p < 0.05$) in

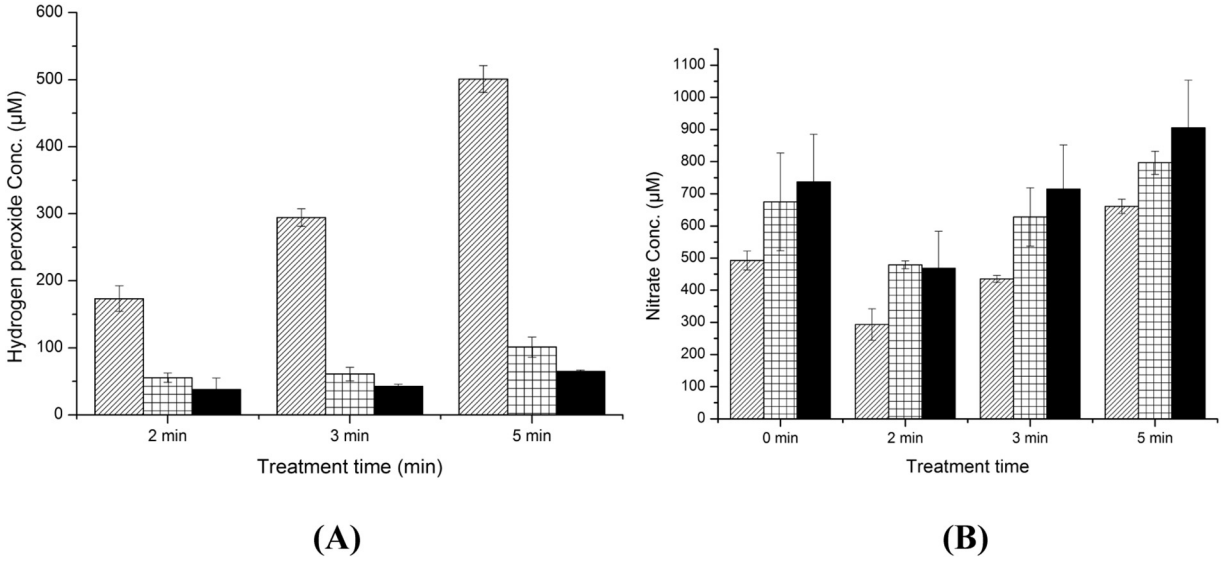

Fig. 5. Generation of (A) hydrogen peroxide and (B) nitrate in dairy effluent after ACP treatment. (▨) 0.2 g/l (▦) 2 g/l (■) 4 g/l.

sensitivity was observed between *B. megaterium* vegetative cells and its spores to ACP treatment. Plasma exposure of 1 min resulted in ±2.50 Log_{10} CFU ml^{-1} reduction in vegetative population while no significant reduction was observed in spore population ($p > 0.05$). Further exposure for 5 min led to complete inactivation of vegetative cells whereas spore population was reduced by ±1.9 Log_{10} CFU ml^{-1} (Fig. 6).

A strong influence of plasma exposure on bacterial spore inactivation efficiency was noted in three different media: PBS, model dairy and meat effluent. The order of bacterial spore's sensitivity to ACP treatment was as follows: PBS > meat effluent > dairy effluent. Based on the finding of this study, ACP treatment for 20 min at 80 kV was required for complete inactivation of bacterial spores suspended in PBS while in case of meat and dairy effluent, despite 2 ± 0.2 Log_{10} CFU ml^{-1} of bacterial spore reduction, a considerable proportion of spores remained viable even after ACP exposure of 20 min (Fig. 7). However, extending plasma treatment time to 30 min yielded comparable results for all media with total inactivation of bacterial spores.

3.7. Safety profile of ACP treated wastewater

The eco-toxicity tests employed in this study include some widely standardized short term methods used to estimate the acute or chronic toxicity of chemical toxicants to aquatic ecology. A difference in sensitivity was observed to ACP treated meat or dairy effluents with different organisms as follows: PLHC-1 > RTG-2 ≥ *Daphnia magna*. The cytotoxicity results expressed as EC_{50} values in Table A (Supplementary material) showed higher dose and exposure time dependent responses, with greater effect when higher concentrations (10–20%) were used.

The untreated dairy and meat wastewater showed toxic effect on both fish cell lines and *D. magna* (except *D. magna* in dairy effluent) with 24 h of exposure. Meat and dairy effluent were plasma treated at 80 kV for 5 and 10 min with 10 min PTRT. Concentration and treatment time dependent cytotoxicity of plasma treated effluent was observed in both cell lines where higher toxicity (i.e. >50%) was observed for concentrations above 10% (Figs. 8 and 9).

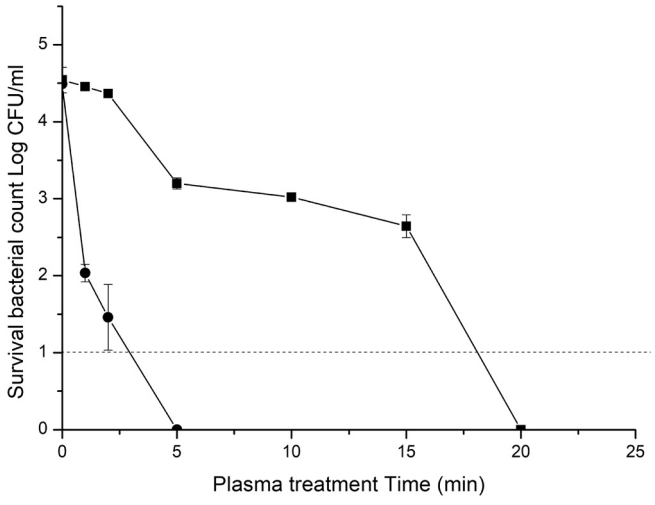

Fig. 6. Time depended inactivation of *B. megaterium* (●) vegetative cells and (■) spores by ACP treatment at 80 kV. The surviving cells were enumerated as Log_{10} value of colony forming units (CFU) on TSA agar plates. Vertical bars indicated as indicated as error bars. Dotted line indicates limit of detection (1 log_{10} CFU ml^{-1}).

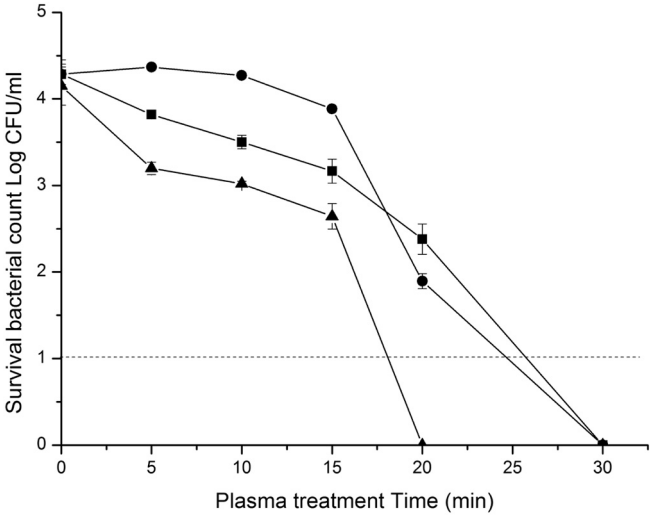

Fig. 7. ACP inactivation of *B. megaterium* spores suspended in (▲) PBS (■) Meat effluent (●) Dairy effluent. Dotted line indicates limit of detection (1 log_{10} CFU ml^{-1}). Vertical bars represent standard deviation.

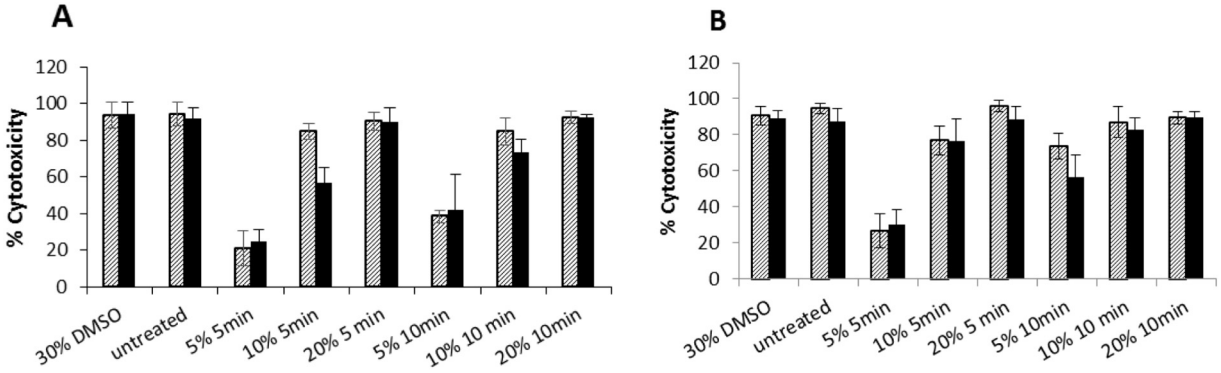

Fig. 8. Percentage cytotoxicity of RTG-2 cells after 24 h exposure to (A) Meat effluent (B) Dairy effluent treated with plasma at 80 kV for 5 and 10 mins. ▨ Alamar blue ▮ Neutral red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The *Daphnia magna* 24 h and 48 h mortality test with ACP wastewater samples is shown in Fig. 10. A 10% effect in Daphnia test (EC_{50}) indicates toxic activity. The EC_{50} values of *D. magna* acute toxicity test after 24 and 48 h exposure to water effluent are shown in Table B (Supplementary material). Following ACP treatment for either 5 or 10 min, the toxicity was reduced by 100% and 73% for a 5% concentration of treated effluent after 24 h *D. magna* exposure. Effects ranged from slight (26%) to high (100%) dependent on the concentration of ACP treated effluent included. However, significant toxicity for all concentrations ≤10% of ACP treated wastewater was observed after 48 h exposure. Release parameters to yield appropriate dilution of treated wastewaters may be manipulated to exploit the ability of ACP to decontaminate wastewater streams, whilst maintaining a safe eco-toxicity profile for aquatic species.

Research by da Costa et al. (2014) demonstrated chlorine can exhibit acute high toxicity for aquatic organisms; disinfection of the wastewater with 2.5 mg Cl_2 l^{-1} resulted in toxicity to cladocerans and fish. According to Watson et al. (2012), the combined activity of residual chlorine and variety of disinfection by products left in the solution further contribute to toxicity in an aquatic system. Petala et al. (2008) evaluated the efficiency of ozone in wastewater treatment at different endpoints and physiological properties. The ozonation of pre-concentrated samples increased the toxic potential of secondary effluent up to 100% after ozonation with 8.0 mg O_3 l^{-1} for 5 min. The mutagenic activity of the treated effluent was also increased after ozonation with low ozone doses and contact time < 5 min, indicating that the ozone conditions strongly affect the formation of ozone-by-products. Despite the increased interest in the development of the non-thermal oxidative processes (corona plasma discharge, UV combined H_2O_2) for decontamination purposes there is very little research done on potential toxicological issues associated with the use of these new technologies. In particular, ACP is considered as an efficient method for degradation of

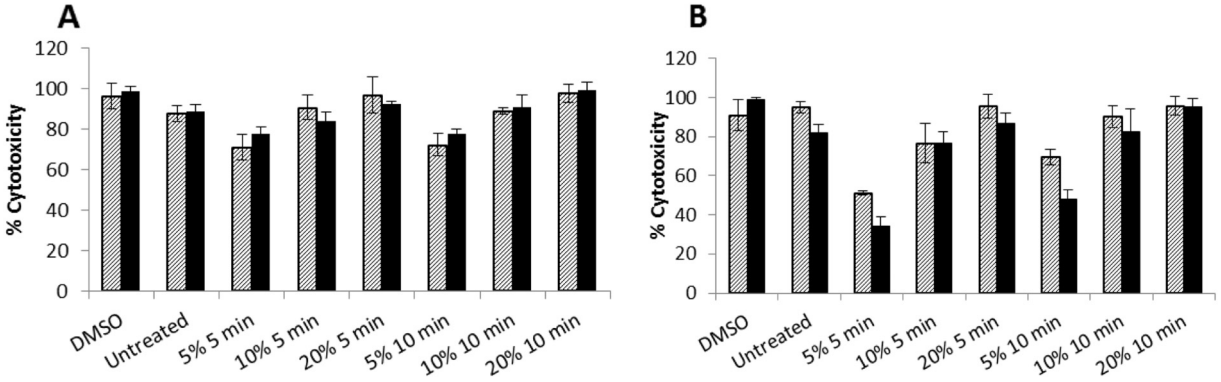

Fig. 9. Percentage cytotoxicity of PLHC-1 cells exposed to different concentrations of (A) Meat effluent (B) Dairy effluent treated with plasma at 80 kV for 5 and 10 mins. ▨ Alamar blue ▮ Neutral red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

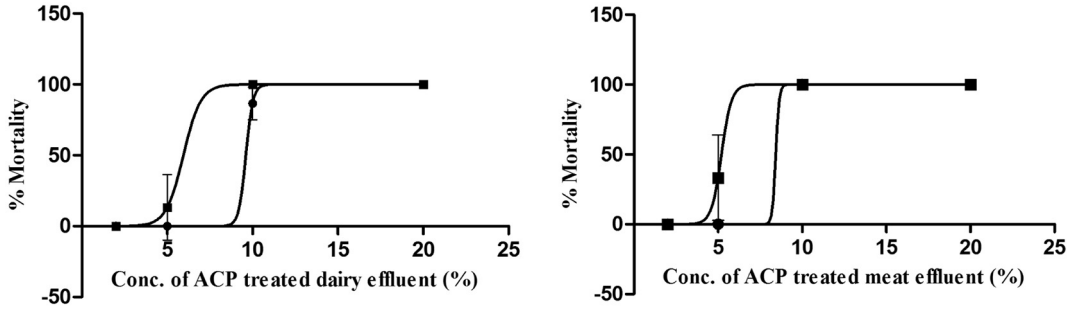

Fig. 10. Percentage mortality or immobilization after 48 h exposure to water effluent plasma treated using DBD-120 system at 80 kV for (●) 5 min (▮) 10 min with 10 min of PTRT.

a wide variety of organic pollutants, pesticides and for microbial control. The presence of nitric and nitrous acids drastically reduces the pH of the test sample which plays an important role in bacteria inactivation but may also play a role in toxicity(Ikawa et al., 2010). Additionally, the intracellular pH in bacteria plays a major role in cell function which affects its enzyme activity, reaction rates, protein stability as well as structure of nucleic acids (Bourke et al., 2017). Studies by Hayes et al. (2013) demonstrated considerable cyto- eco- toxic end points that varied widely depending on treatment parameters and gas used for the plasma treatment. The hydrogen peroxide generated from plasma exposure plays pivotal role in cytotoxicity in combination other plasma-generated species such as peroxynitrite, peroxynitrate (Boehm et al., 2017). Several reactive species and biological relevant factors are generated in plasma discharge, which differ in response to plasma device and treatment conditions. In the current study, plasma treatment of the meat and the dairy effluent was a function of controllable parameters and was efficient in inactivating the key bacterial populations. However, slight toxicity to biological species tested was found. Manipulation of release rate and patterns could present a means to exploit the advantages of ACP for food sector effluent treatment for microbiological safety and reduction of eco-toxicity of effluents, whilst preventing longer term eco-toxic effects. Mecha et al. (2016) showed the toxicity of the treated effluent highly depended at which the treatment of wastewater was applied i.e. raw effluent, primary or after secondary treatment and species tested. Removal of organic and inorganic substances from the wastewater significantly reduced the toxicity level and enhanced biodegradability.

4. Conclusion

In conclusion, the study showed proof-of-principle on safe treatment of food sector wastewater effluents using ACP for decontamination, with useful efficacy within short periods of both treatment and retention times. The efficiency of ACP varies with experimental parameters, target effluent composition and the physiological state of the microorganism. ACP treatment was shown as a promising technology for reduction and complete inactivation of key indicator microorganisms in model dairy and meat wastewater effluent. Depending on the microorganism target, 5 min of ACP treatment at 80 kV with 10 min of PTRT was sufficient for effective elimination of monoculture bacteria in both model effluents while mixed culture challenges posed greater resistance to ACP treatment. This was overcome by simply prolonging the treatment time which effectively eliminated all bacterial strains from dairy effluent. Based on the results and process parameters obtained from this study using lab scale DBD system, ACP shows potential for scale up to larger industrial installations. Retention time and treatment time emerged as critical parameters in bacterial inactivation efficiency by ACP, which is important for design and development of efficient wastewater treatment solutions.

Whilst ACP treatment reduced the toxicity of the food sector effluents up to 24 h exposure, a prolonged contact of up to 48 h was toxic to Daphnia. The test species selected as eco-toxicity biomarkers are considered sensitive organisms and may not mimic environmental conditions closely enough to accurately predict anthropogenic contamination. Therefore, it is recommended to employ a wider variety of biological test species for conclusive results in addition to variable ratios of treated effluent concentrations and times to progress how this technology can be safely deployed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.02.269.

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