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Evaluation of cytotoxicity, genotoxicity, and apoptosis of wastewater before and after disinfection with performic acid

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ABSTRACT

Disinfection with performic acid (PFA) represents an emerging technology in wastewater treatment. Many recent studies indicate its effectiveness and suitability as a disinfectant for different applications; several have demonstrated its reliability as an alternative to chlorine for disinfecting secondary effluents from urban wastewater treatment plants (WWTPs). Some disinfection technologies, in relation to their oxidative power, lead to the formation of disinfection by-products (DBPs), some of which are of concern for their toxic and carcinogenic potential. The aim of this study was to investigate potential genotoxic, cytotoxic, and mutagenic effects of this disinfection agent on treated secondary effluent coming from a municipal WWTP. A strategy with multiple short-term tests and different target cells (bacterial, plant, and mammalian) was adopted to explore a relatively wide range of potential genotoxic events. The Ames test (point mutation in *Salmonella*), the micronucleus (chromosomal damage) and Comet tests (primary DNA damage) on human hepatic cells (HepG2) were conducted to detect mutagenicity and chromosomal DNA alterations. DNA fragmentation and mitochondrial potential assays were conducted to evaluate apoptosis in the same kinds of cells. Mutagenic and clastogenic effect potentials were evaluated by examining micronucleus formation in *Allium cepa* root cells. In all the *in vitro* tests, carried out on both disinfected and non-disinfected effluents, negative results were always obtained for mutagenic and genotoxic effects. In the *Allium cepa* tests, however, some non-concentrated wastewater samples after PFA treatment induced a slight increase in micronucleus frequencies in root cells, but not in a dose-related manner. In conclusion, PFA applied for disinfection to a secondary effluent from a municipal wastewater treatment plant did not contribute to the release of genotoxic or mutagenic compounds. Further studies are required to establish to which extent these findings can be generalized to support PFA for other disinfection applications.

1. Introduction

The disinfection of wastewater effluent represents a fundamental treatment to guarantee the discharge quality and minimise potential health risks to humans and the environment. This treatment is especially essential when the discharge is near beaches or is used directly or indirectly for agricultural or other critical purposes, such as domestic or recreational use. Moreover, because water reuse is a main component of water conservation, attention to potential health risks coming from wastewater effluent is high and will probably increase in the future (Huertas et al., 2008).

All disinfection systems, particularly those that include chemical processes, can produce disinfection by-products (DBPs) from oxidative reactions with substances occurring in water; these can cause adverse effects at different trophic levels in aquatic ecosystems and directly or indirectly in humans. More than 600 DBPs have been reported for the major chemical disinfectants, including chlorine, ozone, chlorine dioxide, chloramines, and their combinations in water treatment (Krasner et al., 2006; Richardson et al., 2007). Some of these compounds, such as chlorinated organic DBPs,

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are of concern because their level in drinking water is associated with several types of cancer occurrences (Leong et al., 2008).

In a review of 30 years of research on the occurrence, genotoxicity, and carcinogenicity of DBPs in drinking water, Richardson et al. (2007) highlighted that the levels of many emerging DBPs, still not regulated in the USA, increased when alternatives to chlorination (primarily ozone or chloramines) were used, and many were more genotoxic than some currently regulated DBPs.

Peracetic acid (PAA), one of the main alternatives to chlorine for wastewater disinfection that has emerged over the last 30 years, is known for its low probability to form DBPs (Liberti and Notarnicola, 1999; Leong et al., 2008; Luukkonen and Pehkonen, 2016). However some studies using PAA, sometimes under extreme conditions, have reported halogenated organic compounds and aldehydes production (Booth and Lester, 1995; Karpova et al., 2013; Dell'Erba et al., 2007) and some mutagenic and genotoxic effects in bacteria and *Allium cepa* respectively (Monarca et al., 2000) for this chemical.

Even ultraviolet light (UV), which is known as a "clean" technology, has the potential to produce aldehydes (Leong et al., 2008) or by-products depending on the lamp type (Linden et al., 2007).

Among disinfection technologies, performic acid (PFA) has emerged in recent years as a reliable alternative to chlorine for its effectiveness and relatively few qualitative impacts. PFA is reported to be a wide-spectrum disinfectant that can inactivate viruses, bacteria and bacterial spores, mycobacteria, and microscopic fungi (Gehr et al., 2009), and it is used in surgery, chemistry, medicine, and the food industry (Rutala and Weber, 2001; Gehr et al., 2009; Heinonen-Tanski and Miettinen, 2010). Since its applicability and reliability in wastewater disinfection were documented (Ragazzo et al., 2007), PFA has been studied for numerous applications. It was more effective than PAA and UV for advanced primary effluent disinfection (Gehr et al., 2009), more effective than PAA and chlorine hypochlorite (HYP) in secondary effluent disinfection at batch and full scales (Karpova et al., 2013; Ragazzo et al., 2013), and a more potent disinfectant than PAA and perpropionic acid for tertiary wastewater (Luukkonen et al., 2015). Furthermore, for the very short contact time required for effectiveness, it was even proposed for disinfection of overflow from overloaded combined sewage systems (Chhetri et al., 2014, 2015).

Although studies related to the qualitative impacts of PFA are limited, a few refer to the weak ability of this peroxyacid to oxidise organic matters, pharmaceutical compounds, or endocrine disruptors (Gagnon et al., 2008; Ragazzo et al., 2013; Luukkonen et al., 2015), which may suggest a low tendency to form by-products. Furthermore, no toxicological effects on *Vibrio fischeri* were detected, nor were aldehyde or bromate formation, nor volatile organic compounds variation from treated and untreated effluent, under full-scale conditions at PFA dosages between 0.3 and 2.4 mg/L (Ragazzo et al., 2013). The formation of brominated phenols and acetic acids, documented by Veijalainen et al. (Veijalainen, A.-M., Heinonen-Tanski, H., Tarhanen, J. 2009, unpublished report) in laboratory tests with synthetic wastewater spiked with high bromide concentrations (101 mg/L), was not confirmed in full-scale trials with bromide concentrations around 1 mg/L (Ragazzo et al., 2013). To date, little is known about cytotoxic and genotoxic properties of PFA and its potential to form toxic by-products.

The aim of this study was to investigate the genotoxic and mutagenic properties of municipal wastewater treatment plant (WWTP) effluent treated with PFA. A combination of genotoxicity tests affecting different endpoints (point mutation, chromosomal mutation, and DNA damage) in different cells (bacterial, plant, and human) was performed. The *Salmonella*/microsome assay (Ames test) is the most commonly used mutagenesis test for routine chemical testing, and it is widely used in studies of disinfection effects in water. It is a sensitive test for detecting point mutations in bacteria, and evidence of mutagenic activity suggests that a substance might be carcinogenic (APHA et al., 2012). Owing to its high reliability at predicting carcinogenicity of chemicals (Benigni and Bossa, 2011), it is valued as preliminary screening tool to determine the mutagenic potential of substances (OECD, 1997; Claxton et al., 2010; APHA et al., 2012).

Plants can detect a broad spectrum of compounds and can be excellent indicators of the cytogenetic and mutagenic potential of environmental pollutants (Leme and Marin-Morales, 2009; Ma et al., 1995). *Allium cepa* has been widely used to evaluate chromosomal damage and disturbance in the mitotic cycle, and it is particularly suitable for genotoxicity studies of surface waters and wastewater (Papa et al., 2016; Bertanza et al., 2013; Leme and Marin-Morales, 2009). Owing to a mixed function oxidase system in its roots cells, it can also detect promutagens (Fatima and Ahmad., 2006).

A combination of a bacterial reverse mutation test (such as the Ames test) and the micronucleus test on mammalian cells is recommended by the European Food Safety Authority (European Food Safety Authority EFSA, 2011) for *in vitro* genotoxicity testing, as they can detect all three genetic endpoints: gene mutation (Ames), and structural and numerical chromosomal alterations (MN). A similar approach has been adopted by the Health & Consumer Protection Directorate-General (EC, 2003) for testing genotoxic properties of xenobiotic metabolites in groundwater. Furthermore, in a recent study (based on the evaluation of 962 rodent carcinogens and *in vivo* genotoxins), the same combination could detect all relevant rodent carcinogens and genotoxins (Kirkland et al., 2011).

Following these recommendations and findings, in the present study, two series of genotoxicity tests on human hepatic cells (HepG2) were performed. HepG2 cells, which maintain many of the morphological characteristics of liver parenchyma, have the advantage of possessing phase I and II enzymes, which play an essential role in the activation and detoxification of promutagens/procarcinogens; they are particularly suitable in mutagenicity studies (Knasmüller et al., 1998). The micronucleus test (cytokinesis-block MN test) allows detection of substances causing damage such as chromosome breakage or damage to the mitotic spindle; owing to its reliability and reproducibility, this assay is a standard cytogenetic test for genetic toxicology testing in human and mammalian cells (Fenech, 2007). The alkaline single-cell microgel-electrophoresis assay (SCGE or Comet test), which detects DNA single- and double-strand breaks, alkali-labile lesions, and DNA strand breaks arising during lesion repair, is a sensitive method for measuring even low-level DNA strand breakage in individual cells; it is considered an indicator test for detecting premutagenic lesions and has been proposed for mechanistic studies (Tice et al., 2000). For apoptosis evaluation, DNA fragmentation and mitochondrial potential assays have been conducted on the same kind of cells.

In relation to the relevance of the experiments: bacteria do not have enzymes involved in promutagen activation and the exogenous enzyme mixture *S9* only partially represents mammalian cell metabolism (Knasmüller et al., 1998); plants, not requiring samples concentration which could imply loss of volatile compounds, can detect the whole potential mutagens of wastewater; human hepatic cells may have the highest predictive value for detecting effects that are relevant for humans.

In general, the comprehensive tests system covers different organisms and assays, which are complementary for different classes of environmentally relevant DNA carcinogens and endpoints (Míšik et al., 2011).