Assessment of peracetic acid disinfected effluents by microbiotests

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Supporting information
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Additional comments about the aim of the work

As reported in the 'Introduction', eco-toxicological aspects related to wastewater treatments are often neglected. If the disinfection process is considered, they can be related to the action of the residual disinfectant agent (in the specific case residual peracetic acid, PAA) and of the disinfection by-products (DBPs), formed during the process. The analytical detection of specific compounds in the disinfected effluents cannot detect possible synergistic or antagonistic effects. When toxicity tests are performed on disinfected samples (in which residual PAA is still active), the possible toxic effect is due to all the above mentioned factors. On the contrary, when toxicity tests are performed on samples in which residual active PAA is quenched, it is possible to estimate the possible toxic effect due only to the compounds present in the disinfected effluents, taking into account their interactions. Finally, it is important to highlight that PAA decomposition in acetic acid and oxygen is not immediate, but requires a lag time which is a function of organic and inorganic composition of the water matrix (*13*).

As a consequence of the frame depicted her and in the 'Introduction' Section, the research work is focused on direct and indirect toxicity of PAA: direct toxicity is due to direct action of active PAA; indirect toxicity is due to possible chemical modification of the treated effluent (for example due to DBPs formation and their interactions), independently from the presence of a residual active PAA.

Wastewater used in the experiments

The main characteristics of the two secondary effluents, are summarised in Table 2. The first municipal wastewater treatment plant (MWTP1) is a conventional plant whose treatment scheme includes pre-denitrification/nitrification (suspended biomass), secondary clarification and sand filtration. The second one (MWTP2) includes: primary flocculation and clarification, biological treatment for nitrogen removal (two in-parallel sections based on suspended and attached biomass respectively) followed by a tertiary stage (chemical precipitation, lamella plate settling, sand filtration).

Table SI – S1. Main characteristics of MWTP1 and MWTP2 effluents used for disinfection tests.

		MWTP1 effluent			MWTP2 effluent		
Parameter		#	Mean ± St. error	Min – Max	#	Mean ± St. error	Min – Max
Ph	–	18	7.6±0.35	6.8 – 8.1	18	7.7±0.32	7.2 – 8.5
SST	mg/L	18	0.8±0.93	0.01 – 4.1	18	6.7±2.06	3.5 – 12.0
COD	mg/L				18	40±6.8	28 – 52
TOC	mg/L	8	12.8±3.82	6.9 – 18.0			
Absorbance: [a]	cm^{-1}						
254 nm		17	0.073±0.007	0.063 – 0.092	18	0.302±0.051	0.188 – 0.407
436 nm		10	0.008±0.002	0.005 – 0.012	12	0.035±0.006	0.025 – 0.047
558 nm		11	0.003±0.001	0.002 – 0.005	13	0.023±0.005	0.015 – 0.031
660 nm		11	0.001±0.001	0.000 – 0.004	13	0.010±0.003	0.006 – 0.016

[a] Optical path 1 cm.

Toxicity tests

Microtox test. Microtox test measures the acute toxicity to the marine bacterium *Vibrio fischeri* (Gram negative marine bacteria) as reduction of bioluminescence. Readings of bioluminescent response were measured using a Microtox Model 500 analyser (SDI, USA) and the acute toxicity data were obtained and analysed using the MicrotoxOmni software. EC50 tests were performed at

5, 15 and 30 minutes. Exposure was carried out at 15°C in Microtox® diluent. Tests were performed according to the Basic Protocol (on PAA solutions) in order to obtain a dose/response curve with the maximum level of reliability and repeatability, and to the Comparison Protocol on effluents before and after disinfection at bench scale.

Rapidtoxkit. The acute toxicity was also evaluated using the freshwater shrimp *Thamnocephalus platyurus*. This toxicity test (Microbiotest, Mariakerke (Ghent), Belgium) is performed using instars II–III larvae of the crustacean, which are hatched from cysts. Hatching needs 30-45 h prior to the start of the test, at 25°C under continuous illumination of 4,000 lux. Upon hatching, the crustaceans were exposed to the samples to be tested for 60 minutes at 25°C. After that they are fed with a natural red coloured suspension and kept in contact with the tested sample for 30 minutes at 25°C. The final observation is carried out by a stereomicroscope and is focused at counting the number of individuals which have assumed food and are thus red from the ones which did not and are thus non coloured. The result, in terms of inhibition, is expressed by comparing the percent of coloured individuals in the tested samples and in the control.

Daphtoxkit F™ magna. Acute toxicity immobilization tests were performed on Daphnia magna neonates that were less than 24 h old. The kits (Microbiotest, Mariakerke (Ghent), Belgium) contain "dormant" eggs (ephippia) which can be hatched on demand. Neonates of uniform characteristics and ready for use can be obtained in 72 h in standard aerated freshwater, at 20-22°C, under continuous illumination of 6,000 lux. Four replicates were tested for each sample and five neonates were used in each replicate. After 24 h and 48 h incubation, at 20°C in the dark, the number of dead and immobilized test organisms in the tested samples and in the controls (standard freshwater) was counted. Organisms are considered immobilised when no independent movement can be visually observed after gentle agitation of the test liquid for 15 s. The test is valid if the number of dead + immobile organisms in the controls does not exceed 10%. EC50 is calculated on the basis of percent inhibition in the tested samples versus controls.

Algaltoxkit F™. The Algaltoxkit F. is a 72 h assay based on growth inhibition of the micro-algae, with calculation of the 72 h EC50 (Microbiotest, Mariakerke (Ghent), Belgium). The kit contains all the materials, including the test organism *Selenastrum capricornutum* (first renamed as *Raphidocelis subcapitata* and presently as *Pseudokirchneriella subcapitata*) immobilized in algal beads. The tests can be started within 30 minutes after deimmobilization of the microalgae from the beads. The algal cells are incubated in the tested samples, enriched by the needed nutrient salts, and in the Standard culture medium for 3 days at 21-25°C, with constant uniform illumination (10,000 lux for sideway illumination or 3,000-40,00 lux for bottom illumination). The optical density at 670 nm (OD) is used as the parameter for algal density. The tests are performed in disposable cells of 10 cm path-length which allow for direct and rapid scoring of the OD in the "long cell" test vials, using any conventional spectrophotometer equipped with a holder for 10 cm cells. The inhibition is calculated by comparing the growth rate in the tested samples and in the standard culture medium.

Additional comments on the experiments

Toxicity tests have been performed, according to the specific aim, on PAA solutions prepared using the culture media as dilution matrix (specific for each test organism) or on wastewater samples, prior and after disinfection. Since all the liquid matrices are characterised by a buffer capacity (due to its own composition), pH variation due to the addition of PAA was negligible, especially in wastewater samples in which alkalinity is usually high. pH values have been periodically measured during the experimentation and, as expected, no significant difference from the neutrality range was observed.

Influence of physical and chemical characteristics of the effluents

Working with real matrices implies some drawbacks, among which the variability of the reference conditions, in the specific case the composition of each sample of the wastewater used during the experimentation (as shown in Table SI –S1). However, the aim of the work was to give general value and effectiveness to the results of eco-toxicological tests, related to the two types of effluents (a domestic and an industrial wastewater) used during the experiments, independently from the presence of specific single compounds (particularly considering that real wastewater changes continuously), but having defined average characteristics.

It is important to underline that:

- the variability of the descriptive parameters of the final effluents are usually restricted to a small range, around the targeted values, since the plant layouts are designed in order to fulfil stringent quality requirements for the final effluents (as in the specific case: MWTP1 effluent has to comply with Italian reuse standards, MWTP2 effluent has to comply with stringent discharge standard since the discharge occurs in lake Como which is considered as a sensitive area);
- the variability range of the data reported in Table 2 (as mean, standard error and variation interval) is not so high if the analytical error of each method is taken into account; this suggests a relative homogeneity of characteristics within each wastewater type (MWTP1 and MWTP2 effluents).

Consequently, the repetition of each type of test several times should help to take into account not only the repeatability of the test response, but also the natural variability of the characteristics of the final effluents, in order to associate a toxic/non toxic effect shown on a test microorganism to an effluent defined by specific (average) characteristics.

In this sense, toxicity data are shown in Table 5 and Figure 3 as average data calculated on the whole set of data coming from the replicates of each type of toxicological tests, instead of effect of PAA disinfection on each sample.

It is again underlined that the focus of the work is a general eco-toxicity assessment of two types of effluents disinfected by PAA, independently from the presence of specific single compounds (particularly considering that real wastewater changes continuously), but with reference to defined average characteristics.