Optimization of low energy sonication treatment for granular activated carbon colonizing biomass assessment

G. Saccani[a], M. Bernasconi[b] and M. Antonelli[a*]

[a]*Environmental Section, Department of Civil and Environmental Engineering (DICA), Politecnico di Milano, Piazza Leonardo da Vinci 32, 20133 Milan, Italy;* [b]*Metropolitana Milanese Spa, Acquedotto di Milano, Servizio Idrico Integrato, Via Meda 44, 20141 Milan, Italy*

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1. Introduction

In drinking water treatment, granular activated carbon (GAC) filters have been used for decades to remove, by adsorption, organic micro-pollutants as pesticides, solvents and taste and odour compounds. It is now well known that GAC can support bacterial colonization and that a large variety of degradation processes take advantage of the amount of organic compounds stored in GAC, with the final result of a longer life of activated carbon.[1] As a drawback, a fraction of attached bacteria are leaked in the effluent during operation or during backwashing. Released bacteria are reported to be 1% ÷ 8% of colonizing cells.[2]

Microbiological presence can be evaluated by different analytical techniques, among which the heterotrophic plate count (HPC) is the most applied,[3–5] with different incubation temperatures and times. Despite their usefulness as reference for normative compliance, plate count methods present the great limitation of determining only the bacterial fraction able to grow in the selected culture medium and incubation conditions.[5–7] For this reason some cultivation-independent methods have been more and more applied in the last few years. Microscopy or flow cytometry total direct count and ATP (adenosine tri-phosphate) determination are mainly reported as a valid alternative to HPC for simplicity, short time required and effectiveness in the quantification of total cell concentration.[4–6,8–11] Flow cytometry and fluorescence microscopy, in particular, are usually coupled with a double staining that allows to determine viable and membrane-compromised cell content as a function of membrane permeability.[5,8] In the staining protocol reported by Barbesti et al.,[8] for instance, SYBR Green I (SG-I) and propidium iodide (PI) are used, respectively, as permeant and non-permeant stain. Consequently SG-I can diffuse only into viable cells giving a green fluorescence, while membrane-compromised cells (damaged cells) are identified due to the co-penetration of PI within the cell and the consequent SG-I fluorescence coverage (shown as PI red fluorescence).

Since the analytical techniques above presented have to be performed on liquid samples, a detachment pre-treatment is necessary when the contribution to microbiological risk due to GAC-colonizing biomass has to be evaluated. So far chemical and physical detachment methods are studied, with sonication as the most effective.[4,10,12–14] Sonication can be classified in high energy sonication (HES) and low energy sonication (LES) in the function of specific

*Corresponding author. Email: manuela.antonelli@polimi.it

energy applied. HES bacterial damaging effects are well known, as this treatment is also used for sludge disruption [15,16] and bacteria inactivation,[17] and are reported to require a bulk substitution procedure to attain the maximum recovery efficiency of viable bacteria.[4] On the contrary, ultrasound damaging effects are not considered when LES treatments are applied on different support media for various exposure times with any bulk substitution.[13,18–20]

This study is aimed at evaluating the effects of ultrasound exposure on bacterial cell viability; consequently a LES treatment for bacteria detachment from GAC particles was optimized. Conventional HPC and fluorescence microscopy with a double staining were used to evaluate cell viability, comparing two LES procedures, without and with periodical bulk substitution.

2. Materials and methods

2.1. GAC adsorption treatment plants

GAC samples (CECARBON GAC 1240, Arkema, main characteristics reported in Table 1) were collected from two adsorption filters in two drinking water treatment plants (plant 1 and plant 2), whose main characteristics are reported in Table 2. Adsorption phase influent and effluent water samples were collected far from backwashing cycles and analysed (four samples) for pH, temperature, dissolved oxygen, HPC of psychrophilic bacteria (22°C incubation temperature) and indicator bacteria (*Pseudomonas aeruginosa*, Coliforms at 37°C, *Escherichia coli* and Enterococci).

Table 1. Main characteristics of analysed GAC (CECARBON data sheet).

Characteristic	Value	Determination method
Origin	Mineral	–
Moisture content	≤2%	ASTM D 2867
Iodine number	1050 mg/g	ASTM D 4607
Surface	1100 m^2/g	N2 BET
Particle's mean diameter	1.6 mm	ASTM D 2872
Particle's fraction ≥1.70 mm (12 mesh)	≤3%	ASTM D 2862
Particle's fraction ≤0.425 mm (40 mesh)	≤4%	

2.2. LES treatment optimization tests

Detachment optimization tests by sonication were performed on GAC samples dipped in phosphate-buffered saline solution (PBS, composition: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1.0 L sterile water), with a solid to liquid ratio of 1 g_{GAC} (wet weight) to 5 mL_{PBS} in a 50 mL Falcon tube. Samples were subjected to sonication without and with bulk substitution. Tests without bulk substitution considered 2, 4, 5, 6, 10, 12 and 20 min sonication times; while during tests with bulk substitution, samples were treated for subsequent sonication cycles of different durations (5 min as maximum), which corresponded to GAC global treatment times of 4, 6, 10, 15 and 20 min. Sonication was performed with Transsonic T460/4 Elma (2.5 L, 170 W, 35 kHz). After each sonication time, the bulk was mixed at 2000rpm for 15 s before proceeding to sample collection for microbiological analysis and bulk substitution, when applied. Each LES test was repeated three times.

Bulk obtained by sonicated GAC without bulk substitution was analysed in duplicate for psychrophilic (22°C) HPC and total and viable cell counts.

Bulk obtained by sonication with bulk substitution was analysed in duplicate by fluorescence microscopy and indicator bacteria enumeration (*P. aeruginosa*, Coliforms at 37°C, *E. coli* and Enterococci).

2.3. Analytical methods

HPC of psychrophilic bacteria was determined by the spread plate method, according to ISO standard UNI EN ISO 6222:2001 with an incubation time of 3 days. Psychrophilic HPCs performed on bulk samples were also determined after 7 days of incubation.

Total cell count was performed by fluorescence microscopy with two different fluorochromes: SG-I (1:10,000v/v, Molecular Probes, Eugene, OR) and PI (concentration of 10 μg/mL, Sigma, St. Louis, MO). The method applied

Table 2. Main characteristics of the groundwater treatment plants, and measured values for influent and effluent water characterization analyses.

				Influent	Effluent
	Plant characteristics		Influent and effluent water characteristics		
Plant 1	No. of treatment devices	21 filters in parallel	DO (mg/L)	5.1 ± 0.78	1.2 ± 0.35
	Specific flow (L/s)	15	*T* (°C)	15.9 ± 0.14	15.3 ± 0.21
	Specific rate (m/h)	11	pH	7.8 ± 0.08	7.8 ± 0.12
	Specific EBCT (min)	11	HPC 22°C (cfu/mL)	13.5 ± 15.80	6.0 ± 3.16
Plant 2	No. of treatment devices	20 filters in parallel	DO (mg/L)	15.3	4.7
	Specific flow (L/s)	25	*T* (°C)	15.2	6.1
	Specific rate (m/h)	13	pH	7.6 ± 0.05	7.7 ± 0.05
	Specific EBCT (min)	10	HPC 22°C (cfu/mL)	4.3 ± 1.53	3.0 ± 1.41

Note: EBCT, empty bed contact time.

is the one optimized by Barbesti et al. [8] which allows to distinguish between viable and membrane-compromised cells by cellular membrane permeability, providing total cell count as the sum of viable and damaged cells.

P. aeruginosa was analysed by the filtering membrane method (UNI EN ISO 12780:2002), while the contents of Coliforms at 37°C, *E. coli* and Enterococci were determined with the Defined Substrate Test method [21] respectively according to Italian standards ISS A006A, ISS A001A and ISS A002A,[22] as most probable number of bacteria per 100 mL (MPN/100 mL).

Dissolved oxygen and temperature were measured by an Oxi 340i/SET (WTW) probe according to standard methods for the examination of water and wastewater[23]; pH was determined with an automatic titration system (MAN-TECH) according to Italian standards.[24]

2.4. Statistical analyses

Statistical analyses were performed by SPSS 16.0 for windows. Monitoring data belong to the supplied water quality database, composed of more than 100 chemical parameters analysed at different wells of the supplying watershed, from 2002 to 2010 (approximately with monthly frequency).

3. Results and discussion

3.1. LES treatment conventional tests

Bulk samples obtained by GAC LES treatment tests without bulk substitution were characterized by HPCs and total direct counts shown, respectively, in Figures 1 and 2.

As shown in Figure 1, psychrophilic HPCs showed relevant standard deviations (SDs) comparable with mean values (77 ÷ 117% of mean value), highlighting a relevant dispersion of data as typical for HPC analyses.[25,26] Comparing HPC's data it has been observed that a different incubation time (3 or 7 days) did not affect observed biomass, both for plant 1 (ANOVA sig. 0.495, Fisher's F 0.469) and plant 2 (ANOVA sig. 0.966, Fisher's F 0.002). Moreover HPC results reveal a greater bacterial population colonizing plant 2 GAC samples, as significant difference for each sonication time has been observed both for 3days (ANOVA sig. 0.000, Fisher's F 23.193) and 7 days incubation time counts (ANOVA sig. 0.000, Fisher's F 19.722). Considering the effect of the applied treatment, HPCs showed decreasing mean values for sonication times higher than 5 min, with lower treatment times providing no significant difference on biomass recovery. Finally, based on maximum obtained detachment (2 min sonication HPC mean values), plant 1 GAC-colonizing biomass correspond to 3.3×10^4 cfu/g_{GAC}, while a higher mean value of 7.1×10^4 cfu/g_{GAC} has been observed for plant 2.

Considering fluorescence microscopy bacterial counts, data presented in Figure 2 reveal lower SDs (23% ÷ 73% of viable cell count mean value and 29% ÷ 91% of total cell count mean value). Observed viable and total cell counts showed a viable cell fraction of 59% ± 14.3% for plant 1, higher than the plant 2 values, equal to 39% ± 20.8%. In terms of mean values, a significant difference between the two GAC-colonizing biomasses has been observed for viable cell counts (ANOVA sig. 0.008, Fisher's F 7.199), while no difference can be seen in terms of total cell counts (ANOVA sig. 0.161, Fisher's F 2.003). Thus, direct cell counts revealed a higher GAC-colonizing active biomass in plant 1 samples in opposition to what was observed by HPCs. Furthermore a relevant difference has been found between direct cell counts and HPCs also in terms of

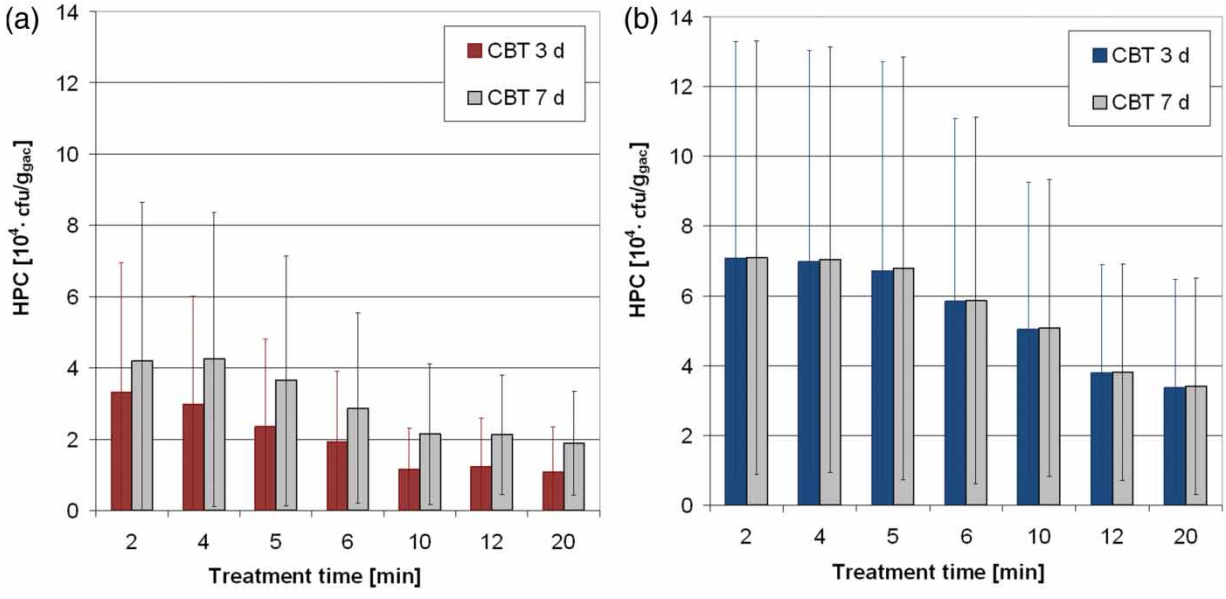

Figure 1. Psycrophilic HPCs of biomass detached from GAC of plant 1 (a) and plant 2 (b), by LES without bulk substitution (mean value ± SD).

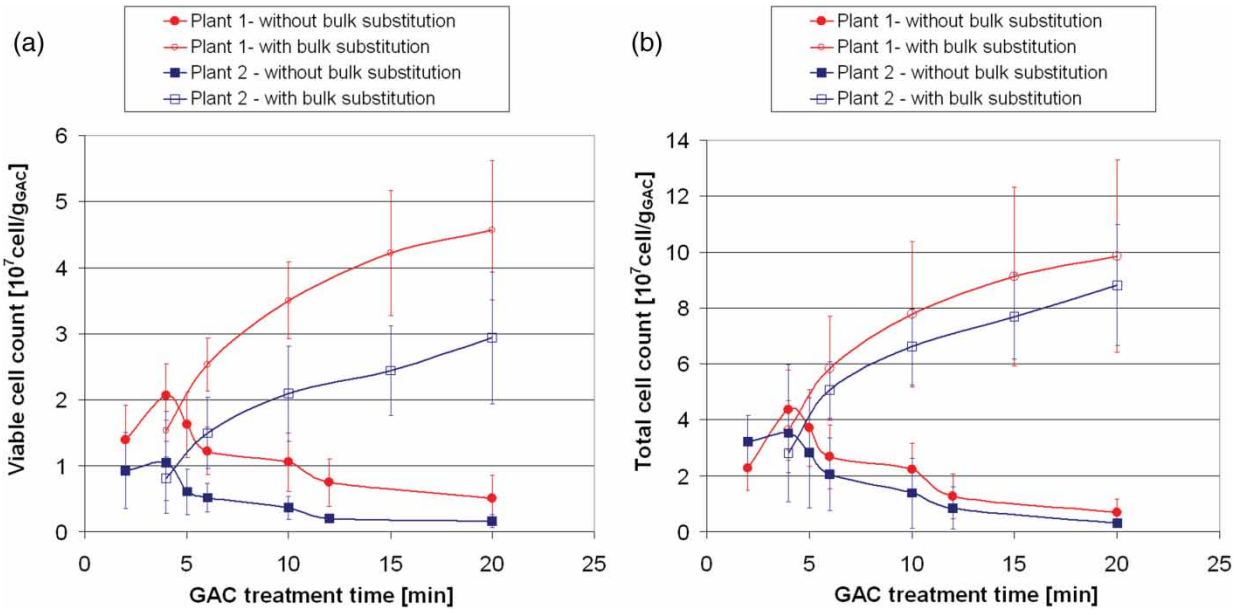

Figure 2. Viable (a) and total (b) cell counts of biomass detached by LES treatment without and with bulk substitution (mean value ± SD).

counted bacteria: HPC (3 days incubation time) observed bacterial fraction corresponds to 0.1% ÷ 0.3% of viable bacterial counts for plant 1 samples and 0.8% ÷ 2.0% of viable bacterial count for plant 2 samples. These values are in agreement with what is reported in literature.[5,6,25, 27,28] If the above reported results of statistical analyses about incubation time are neglected, and mean values only are taken into account, therefore passing over data variability, it is possible to observe that the application of 7 days as HPC incubation time resulted in increasing numbers of cfu only for bulk obtained from plant 1 GAC samples reaching a value of 4.2×10^4 cfu/g_{GAC} and showed no effect for plant 2 GAC samples. Observed results highlighted a population of slower-growing bacteria on plant 1 samples, but the higher incubation time did not allow the detection of the biomass observed by direct cell counts or the observation of a plant 1 GAC-colonizing biomass greater than that of plant 2. All these remarks gave evidence to a detached biomass underestimation through HPC, as it is affected by cultivation and incubation conditions. In particular, determinations on plant 1 bulk samples revealed a greater underestimation for slower-growing bacteria, as reported by Gibbs and Hayes.[29]

Also considering the effect of the applied treatment, direct cell counts lead to interesting observations: the detachment trend is different from that of HPC, as both viable and total bacterial counts presented maximum values at 4 min treatment time. Direct cell count decrease, observed after 5 min sonication, suggested ultrasound damaging effects on bacterial cells, with three possible different contributions:

- detachment of GAC-colonizing bacteria (either viable or damaged cells),
- damage and inactivation of viable bacteria,
- lysis of detached damaged bacteria.

Data in Figure 2 show a bacterial count decrease after 5min of sonication, giving evidence to an ultrasound damaging effect, similar to that reported by Hua and Thompson [17] and Magic-Knezev and van der Kooij [4] for HES treatments. Bacterial count data were fitted by a first-order kinetic to estimate the damaging effect extent, according to the following equations:

$$\frac{dN}{dt_{us}} = k \cdot N,$$

$$\ln(N) = k \cdot t_{us} + \ln(N_{max}),$$

where N = observed cell count (cell/g_{GAC}), t_{us} = GAC ultrasound treatment time (min), k = damaging effect coefficient (cell/min), N_{max} = maximum observed cell count (cell/g_{GAC}), which is considered equal to the cell count observed after 4 min sonication.

The first-order kinetic has been applied to viable, damaged and total cell counts, obtaining the statistics reported in Table 3. Sonication caused a total cell decrease rate of −9.0% cell/min (plant 1 samples) and −11.2% cell/min (plant 2 samples). Viable bacteria inactivation rate (6.5 ÷ 7.9% cell/min) is in agreement with the results of 0.6 ÷ 10.1% cells/min reported for a mixed microbial community from GAC treated by HES,[4] and also with the inactivation rate values observed by Hua and Thompson [17] on *E. coli* cultivations (7 ÷ 10% cell/min), suggesting that the damages are independent from the intensities of sonication, in the studied range. Besides, data show that ultrasound damaging effect is even higher on already membrane-compromised cells, with rate values of 11.5%

Table 3. First-order kinetic coefficient of ultrasound damaging effects model estimated from bacterial direct counts obtained by LES treatment without bulk substitution.

	Plant 1						Plant 2					
	Viable cells		Damaged cells		Total cells		Viable cells		Damaged cells		Total cells	
	k	$\ln(N_{4\,min})$	k	$\ln(N_{4\,min})$	K	$\ln(N_{4\,min})$	k	$\ln(N_{4\,min})$	k	$\ln(N_{4\,min})$	K	$\ln(N_{4\,min})$
Coeff. value (% cells/min)	−6.5%	17	−13.1%	17	−9.0%	17.7	−7.9%	16.1	−11.5%	16.9	−11.2%	17.4
Standard error (% cells/min)	1.61%	0.18	2.41%	0.27	1.79%	0.20	2.28%	0.25	2.96%	0.32	2.45%	0.27
Number	37	37	37	37	37	37	41	41	40	40	41	41
R^2	0.317	0.317	0.456	0.456	0.418	0.418	0.237	0.237	0.285	0.285	0.351	0.351
t	−4.029	95.469	−5.418	63.772	−5.011	89.7	−3.481	63.91	−3.888	53.021	−4.592	64.333
Sig.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

cell/min and 13.1% cell/min. Finally, model results confirmed the coexistence of the three different mechanisms hypothesized (detachment, damage and inactivation, lysis).

3.2. *LES optimization tests*

Direct cell count was found to be a more representative and sensitive analysis to assess ultrasound damaging effect on bacteria, as HPCs were found to be affected by bacterial growth ability at the set incubation conditions. The influence of sonication time on detachment efficiency was then evaluated applying only fluorescence microscopy to bulk samples. Presented results suggested the need for a periodic bulk substitution for the GAC pre-treatment procedure, in order to protect detached bacterial cells from ultrasound damaging effects, applying sonication cycles of 5 min maximum. GAC-colonizing biomass has been determined as the sum of direct cell counts observed in each LES cycle bulk sample. Observed values are presented in Figure 2, as viable and total cell counts obtained with bulk substitution.

Cell counts indicate that biomass values determined after 4 min sonication without bulk substitution represent an underestimation of the GAC-colonizing biomass. After five treatment cycles (corresponding to 20 min sonication of GAC samples) detached biomass was 4.56×10^7 cells/g_{GAC} viable cells and 9.84×10^7 cells/g_{GAC} total cells for plant 1, and 2.94×10^7 cells/g_{GAC} viable cells and 8.81×10^7 cells/g_{GAC} total cells for plant 2. These values correspond to the 222% of viable cells and 226% of total cells of the biomass detached without bulk substitution for plant 1, and 280% of viable cells and 250% of total cells for plant 2. Referring to the biomass detached after 15 min GAC sonication, the fifth treatment cycle allowed a further recovery of (7 ± 1.3)% viable cells and (7 ± 1.2)% total cells for plant 1, and (15 ± 5.2)% viable cells and (12 ± 5.0)% total cells for plant 2, suggesting that it is not worth going on with further sonication cycles. Reported observations revealed that subsequent LES cycles of 5 min maximum treatment time with bulk substitution at the end of each cycle allowed to protect detached bacteria from ultrasound damaging effect, and 20 min GAC LES led to an efficient biomass recovery.

Table 5. Discriminative factor ANOVA, obtained by factor analysis of influent water characteristics.

Factor's index parameter	Sum of squares	df	Mean square	F	Sig.
chromium (VI)	17.815	1	17.815	67.304	0.000
atrazine	2.807	1	2.807	37.484	0.000
TCE	1.558	1	1.558	25.185	0.000
PCE	0.225	1	0.225	11.888	0.002
Freon 11	4.407	1	4.407	10.699	0.003

Cell counts were observed applying sonication with bulk substitution fitted, with the highest R^2 values, to an S-shaped curve (R^2 of 0.877 for viable cell counts and 0.626 for total cell counts in plant 1 samples, and 0.715 for viable cell counts and 0.811 for total cell counts in plant 2). The applied model is described by the equation:

$$N = e^{(b_0+(b_1/t_{us}))}$$

with b_0, b_1 as model parameters.

Data interpolation allowed the estimation of the total GAC-colonizing biomass and the evaluation of the bacterial fraction recovered through the detachment method. Model results are presented in Table 4, where total GAC-colonizing biomass (N_{tot}) has been estimated through the model under the hypothesis of a complete detachment after 40 min sonication. Estimated values are compared to observed values without and with bulk substitution, obtaining recovered biomass fraction calculated from mean observed values, respectively, after 4 and 20 min GAC LES treatment. Recovered biomass fraction values of 87.2% ÷ 100.8% show the effectiveness of the optimized sonication procedure, with the physical meaning of 100.8% recovery value of having attained complete detachment (100%).

Total cell counts of 9.9×10^7 cell/g_{GAC} in plant 1 and 8.8×10^7 cell/g_{GAC} in plant 2 resulted to be 2 log lower

Table 4. GAC-colonizing biomass estimation by LES treatment without and with bulk substitution: total GAC-colonizing biomass estimate (N_{tot}), mean and SD observed values and recovered biomass fraction.

			Without bulk substitution		With bulk substitution	
		N_{tot} S-shaped curve model (cell/g_{GAC})	Observed value (cell/g_{GAC})	Recovered fraction (%)	Observed value (cell/g_{GAC})	Recovered fraction (%)
Plant 1	Viable cells	5.2×10^7	$(2.1 \pm 0.48) \times 10^7$	39.70	$(4.6 \pm 1.05) \times 10^7$	87.90
	Damaged cells	4.6×10^7	$(2.1 \pm 0.93) \times 10^7$	46.20	$(4.6 \pm 2.64) \times 10^7$	100.80
	Total cells	10.8×10^7	$(4.4 \pm 1.43) \times 10^7$	40.50	$(9.8 \pm 3.43) \times 10^7$	91.40
Plant 2	Viable cells	3.2×10^7	$(1.1 \pm 0.77) \times 10^7$	32.50	$(2.9 \pm 1.00) \times 10^7$	90.80
	Damaged cells	5.8×10^7	$(2.3 \pm 1.69) \times 10^7$	39.70	$(5.0 \pm 0.24) \times 10^7$	87.20
	Total cells	9.9×10^7	$(3.5 \pm 2.45) \times 10^7$	35.70	$(8.8 \pm 2.16) \times 10^7$	89.20

than reported in literature for groundwater adsorption filters [4,9]. Furthermore, data reported in Figure 2 and Table 4 show greater viable cell counts in plant 1 (46.3% instead of 33.3% as observed in plant 2 GAC-colonizing biomass). Indicator bacteria enumerations were then performed on bulk samples obtained applying optimized detachment procedure on bigger GAC samples, in order to get the required bulk volume. Coliforms at 37°C were found in all of the four samples analysed (3.1 MPN/100 mL in two samples and 1.0 MPN/100 mL in the others), while *P. aeruginosa* were observed in only one sample (1.0 cell/100 mL). *E. coli* and Enterococci were absent in each sample. These results allow to exclude the presence of pathogenic bacteria in the microbiological population analysed.

3.3. *Influent water characteristics and observed bacterial populations*

Based on data in Table 2, favourable growth conditions for psychrophilic bacteria are suggested by DO, T and pH values, but influent HPCs resulted 2 log lower than both the value of 2.10×10^2 cfu/mL for groundwater and the values of $(15 \div 370) \times 10^2$ cfu/mL for biofilters treating groundwater.[14] Effluent HPC values are directly comparable to influent values, suggesting a negligible biomass release from GAC through filtration, probably due to the low degree of GAC colonization. No indicator bacteria were observed in considered water samples, in agreement with the results obtained analysing GAC detached biomass.

As reported in Sections 3.1 and 3.2, the two plants presented significantly different GAC-colonizing active biomasses. The difference between the two GAC-colonizing biomasses cannot be explained in terms of operating conditions, since they are similar (Table 2). A possible explanation was investigated in influent water contamination, studying supplied water quality monitoring database by multivariate statistical analysis. In particular the correlation between different contamination parameters was studied by factor analysis, and supplied water

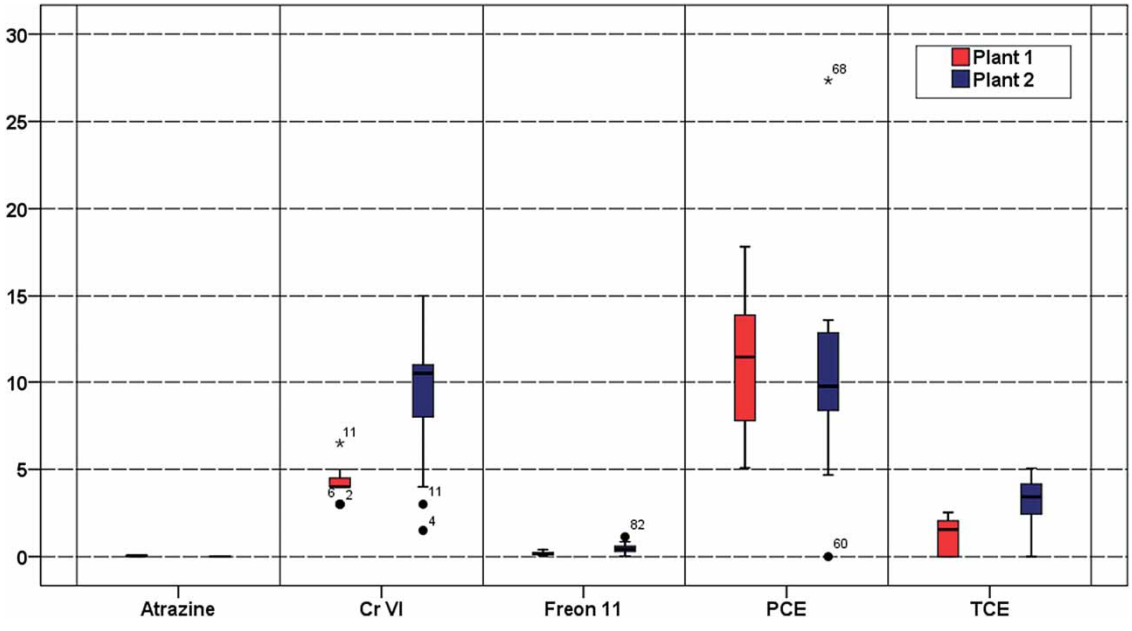

Figure 3. Concentration of discriminative factor's pollutants in considered plant influent water.

contaminations were geo-referred and related to drinking water treatment plants through cluster analysis. Influent water characteristics were found to be significantly different for the two considered plants, with pollutants reported in Table 5 being the most discriminative. Concentrations observed in influent water in the monitoring period, reported in Figure 3, point out a significantly higher presence of chromium VI and trichloroethylene (TCE) in plant 2 influent water. It is, thus, possible to assume a bacterial selection due to the presence of different pollutants, leading to different GAC-colonizing populations. In particular a combined toxic and genotoxic effect is reported by Labra et al. [30] for chromium VI and volatile organic compounds (VOCs) like TCE, consisting in a cellular membrane damaging effect of VOCs that favour the genotoxic effect of chromium VI. These considerations can account for the higher fraction of damaged cells (lower viable cell counts but similar damaged cell counts, as reported in Table 4).

4. Conclusions

Comparing psychrophilic plate counts with viable cell counts by fluorescence microscopy, a cultivable fraction has been observed in the range of $0.1\% \div 0.4\%$ and $0.8\% \div 2.1\%$ for the two considered GAC filters. Plate count method involved a significant underestimation, greater in the case of slower-growing bacteria. Furthermore direct cell count through fluorescence microscopy by the double staining method (SG-I coupled with PI) confirmed a relevant sensitiveness, allowing to determine ultrasound damaging effect on both viable and membrane-compromised detached cells.

As it happens for HES, GAC LES provided bacterial detachment and damaging and inactivation effects at the same time. Observed viable cell inactivation rate was $6.5 \div 7.9\%$ cell/min while ultrasound damaging effect on membrane-compromised cells has appeared to be even higher, with lysis rate values of 11.5% and 13.1% cell/min.

Subsequent LES cycles with bulk substitution at each cycle allowed to protect detached bacteria from damaging and to recover higher fractions of GAC-colonizing biomass. Minimizing ultrasound damaging effects, the optimized detachment treatment consisted in 20 min LES, through subsequent LES cycles of 5 min maximum treatment time, with bulk substitution at the end of each cycle. Optimized LES treatment allowed the recovery of $87.2\% \div 100\%$ of GAC-colonizing biomass, revealing the presence of 8.8×10^7 cell/g_{GAC} of total cells for plant 1 and 9.9×10^7 cell/g_{GAC} for plant 2 even though there was a great difference in terms of viable cells, with values of $(4.6 \pm 1.05) \times 10^7$ cell/g_{GAC} for plant 1 and $(2.9 \pm 1.00) \times 10^7$ cell/g_{GAC} for plant 2. The observed difference was related to a significantly different influent water contamination: a combined toxic and genotoxic effect of chromium VI and TCE can account for the lower bacterial counts observed in plant 2 GAC-colonizing population and for the higher fraction of damaged cells.

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