

SUPPLEMENTARY MATERIAL TO
**Influence of microalgae on organic micropollutants removal
from water by powdered activated carbon**

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Germany. TABLE S1. Results of ICP-OES analysis of metal content in bio-oil samples

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The scheme of experimental methodology is shown in Fig S-1.

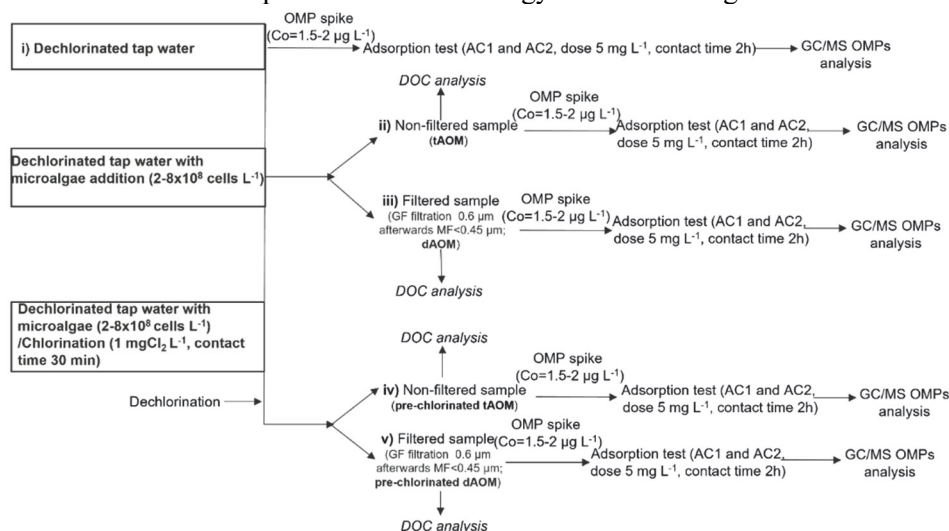


Figure S-1. Work methodology.

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MATERIALS

Powdered activated carbons

Two types of commercial PAC were used for conducting all experiments. According to manufacturer's specifications, AC1 has an iodine number $900 \pm 100 \text{ mg g}^{-1}$, while AC2, which is obtained from carbonized coconut shell, has an iodine number $1000\text{--}1100 \text{ mg g}^{-1}$.

Microalgae selection and cultivation

For this study, two microalgae were selected based on their different morphology and talus: a) *Chlorella vulgaris*, a unicellular green alga, has a spherical shape and size that varies from 2 to 10 μm , and is known for its high lipid content (20–38 %) ^{1,2} and b) *Arthrospira (Spirulina) platensis* with filamentous structure and filaments length from 400–700 μm , ³ known for its rapid growth and high protein content (50–70 %) ^{3, 4, 5}. These differences in morphology and biochemical composition are expected to influence their interaction with micropollutants and PAC and the efficiency of their removal in drinking water treatment processes. The microalgae strains used in this study were obtained from the Novi Sad culture collection of cyanobacteria and microalgae (NSCCC) at the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad. The strains were cultivated under photoautotrophic growth conditions for 15–21 days to reach the exponential phase, and further up to 45 days to reach the stationary phase.

Chlorella vulgaris: Cultivated using commercial sterilized Algae Broth (Sigma Aldrich) under 12 h light and dark cycles and $T = 22\text{--}24^\circ\text{C}$. Medium composition is: 0.05 g L^{-1} of NH_4Cl , 0.058 g L^{-1} of CaCl_2 , 0.25 g L^{-1} of K_2HPO_4 , 0.003 g L^{-1} of FeCl_3 , 0.513 g L^{-1} of MgSO_4 , and 1 g L^{-1} of NaNO_3 .

Arthrospira (Spirulina) platensis: Cultivated for 45 days in sterilized Spirulina-Ogawa-Terui (SOT) (Ogawa T, 1970) medium under 12 h light and dark cycles and $T = 22\text{--}24^\circ\text{C}$. Medium composition is: NaHCO_3 16.8 g, K_2HPO_4 0.5 g, NaNO_3 2.5 g, NaCl 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.04 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g, $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ 0.08 g, trace mineral mix A5 1.0 mL.

The number of algal cells was counted using the standard direct counting method under a microscope (Kern Optic, OBN 135, Germany) with a hemocytometer plate and expressed as cells per L.

Organic micropollutants

For this study, investigated organic micropollutants are ibuprofen (IB), caffeine (CF) and diclofenac sodium salt (DCF), all purity $\geq 99\%$, purchased from Sigma Aldrich. Aqueous stock solutions ($\sim 5 \text{ mg L}^{-1}$) of substances were prepared in distilled water by sonication for 3 h and filtration through a $0.45 \mu\text{m}$ membrane filter (Sartorius, US). They were further diluted and used for spiking the water matrices (i, ii, iii, iv and v, Fig.S1) to achieve nominal concentrations of OMPs

around 1.5-2 $\mu\text{g L}^{-1}$ Table SI summarizes the physico-chemical properties of the selected compounds.

Table S-I. Chemical and physical properties of the analyzed organic micropollutants

Substance	MW ^a (g/mol)	ACD/logD ^b pH=7.4	pKa at 25 °C ^a	Charge at pH 7.4
Ibuprofen (IB)	206.28	0.45	5.3	-1
Diclofenac (DCF)	296.1	1.37	4.15	-1
Caffeine (CF)	194.19	0.28	14	0

^aPubChem 2024⁶; ^bChemSpider, 2024⁷; Abbreviations: MW - Molecular Weight; ACD/logD - Distribution Coefficient at pH=7.4.

Water matrices

Tap water from Novi Sad, Serbia was used for preparation of five water matrices:

i) Dechlorinated tap water without algae addition (DCTW); Dechlorination is performed with solution of Na-sulphite ($C=0.1 \text{ g mL}^{-1}$) in a way that 0.1 mL is added per 5 mg $\text{Cl}_2 \text{ L}^{-1}$ of residual chlorine.⁸ A part of DCTW was taken and spiked with selected OMPs to obtain nominal concentrations of 1.5-2 $\mu\text{g L}^{-1}$. For each matrix enrichment, aqueous solutions of OMPs were used. For each type of microalgae and phase of growth following type of matrices were prepared:

ii) DCTW with total algal organic matter (tAOM); In 12 L of DCTW 80 mL of culture (*A. platensis* or *C. vulgaris*, exponential or stationary phase of growth) was added. After algae addition, solution was slowly mixed for 10 min at 60 rpm and during the mixing three aliquots of 10 mL were taken and mixed as a composite sample in a beaker, afterwards, a composite sample (30 mL) was taken for algae abundance analysis using the standard direct counting method. This way the constant number of algae after repeating the experiment 3 times, was proven to be $2-8 \times 10^9 \text{ cells L}^{-1}$. Then, the whole mixture is transferred into tank filled with 90 L of DCTW and slowly mixed for 10 min at 60 rpm, after which, the abundance analysis is performed again to reach the number of algae of $2-8 \times 10^8 \text{ cells L}^{-1}$, in order to simulate algal abundance characteristic for eutrophic surface water. The solution was left undisturbed at room temperature under 12 h light and dark cycles for 24 hours to allow algae adaptation. After this period, the sample was mixed at 60 rpm for 10 minutes before measuring the algae abundance again. A part of this sample from the tank was used and spiked with selected OMP, in the same way as explained for the previous matrix (i);

Before the experiments, method for measuring the algae abundance was tested five consecutive times to demonstrate measurement consistency. The obtained values are presented in Table SII. These tests confirmed that algal densities consistently ranged from $1-3 \times 10^8 \text{ cells L}^{-1}$. In the main experiments, algal densities varied from $2-8 \times 10^8 \text{ cells L}^{-1}$, likely due to the use of fresh algal cultures in each run. Nonetheless, for future studies, standardizing the initial algal density

across all experiments could enhance methodological consistency and enable more accurate comparisons between different experimental conditions.

Table S-II. Microalgae abundance consistency in five separate test replications.

Number of cells in separate experiments taken from the 100 L water tank after culture addition and adaptation (cells L ⁻¹)	
<i>A. platensis</i>	<i>C. vulgaris</i>
1.0×10 ⁸	2.4×10 ⁸
3.0×10 ⁸	2.1×10 ⁸
2.95×10 ⁸	1.9×10 ⁸
1.2×10 ⁸	3.5×10 ⁸
1.15×10 ⁸	2.0×10 ⁸

iii) DCTW with dissolved algal organic matter (dAOM); Preparation: a part of matrix ii) was filtered through glass-fiber filter (0.6 µm, MACHEREY-NAGEL, Germany) and afterwards through 0.45 µm membrane filter (Sartorius, US), to obtain the solutions with dAOM. After filtration matrix was spiked with selected OMP, in the same way as explained for the matrix (i);

iv) Pre-chlorinated tAOM - a part of the matrix ii) is chlorinated with the dose of 1 mg Cl₂ L⁻¹, and left for 30 min to react and afterwards dechlorinated again based on measurement of actual concentration of chlorine in the samples, and after that spiked with selected OMPs as previously explained, to avoid the contact of chlorine and OMPs;

v) Pre-chlorinated dAOM - a part of the matrix ii) is chlorinated with the dose of 1 mg Cl₂ L⁻¹ and left for 30 min to react and afterwards dechlorinated based on measurement of actual concentration of chlorine in the samples. Sample is further filtrated through glass-fiber filter (0.6 µm, MACHEREY-NAGEL, Germany) and afterwards through 0.45 µm membrane filter (Sartorius, US). Upon filtration it was spiked with selected OMPs.

ANALYTICAL METHODS

Sample preparation and GC/MS OMPs analysis

In an aliquot of the sample (250 mL), the pH value (pH=2) is adjusted using cc of HCl (p.a >37 %, Centrochem). Sample was spiked with the 25 µL of mecoprop internal standard working solution (PESTANAL® analytical 99.6 % (HPLC) Sigma-Aldrich, c=1 µg L⁻¹), and pre-concentrated with the Oasis® HLB (3cc, 60 mg) SPE cartridge (Waters, Massachusetts, USA) according to the following procedure: a) Conditioning – 3x1 mL of mixture ethyl-acetate/methylene-chloride 1:1 (EtAc – Sigma Aldrich, 99 %; DCM for pesticide residue analysis, Chromasolv™, Honeywell), than 3x1 mL of methanol (for pesticide residue analysis, Chromasolv™, Honeywell), and 3x1 mL of acidic laboratory water (pH ≈ 2); b) Sample loading (flow 5 mL min⁻¹); c) Sorbent drying under vacuum for 1h and d) Elution with 3 aliquots of 1 mL EtAc/DCM (1:1) with

20 min retention of the last solvent portion. Further, solvent was removed under N₂ at room temperature. Extracts were quantitatively transferred to vials by washing the walls of the cuvette with 0.5 mL of toluene (for pesticide residue analysis, Chromasolv™, Honeywell). Before that, 1 µL of working solution of the second internal standard for chromatography check, phenanthrene d-10 ($c=0.4 \mu\text{g L}^{-1}$) is added to vial and dried under N₂ and afterwards derivatization was performed using 100 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide MSTFA (Synthesis grade, Sigma-Aldrich) at 60 °C for 1 h.⁹

IB, CF and DCF were measured by GC/MS (Agilent 7890B GC with 5977A MSD). The column Agilent J&W Scientific, DB-5 MS (30 m × 0.25 mm × 0.25 µm; Agilent, USA) was applied with helium as the carrier gas in constant flow (1 mL min⁻¹). 2 µL of the extract was injected in the splitless mode at 250 °C (purge flow 30 mL min⁻¹ at 0.75 min). Temperature program applied is 100 °C–2 min; 15 °C min⁻¹ up to 180 °C; 30 °C min⁻¹ up to 230 °C which was held 5 min and afterwards 20 °C min⁻¹ up to 270 °C which was held 3 min. Method characterization is shown in Table SIII.

Table S-III. GC/MS method characterization

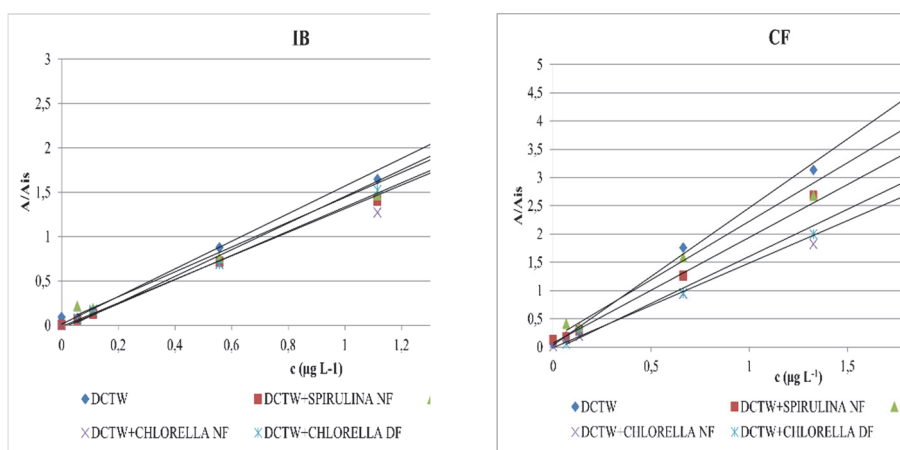
OMP	Target ion, qualifier ion	Calibration		Method repeatability, RSD, %		Instrument repeatability, RSD, %		Bias %		MDL*	PQL**
		Range (µg L ⁻¹)	R ²	at 0.5	at 1.5	at 0.5	at 1.5	at 0.5	at 1.5		
IB	160, 263, 117	0.05-2.12	0.999	3	4	5	2	8	2	0.02	0.08
CF	194, 109, 82	0.05-2.01	0.994	2	6	2	4	0.3	6	0.03	0.091
DCF	214, 242, 367	0.05-1.89	0.995	10	2	2	2	3	4	0.024	0.071

*MDL- method detection level. **PQL- practical quantitation level

Calibration was done in DCTW in triplicate and average values are presented in Table SIII. MDL is calculated from the standard deviation (SD) of seven replicates in DCTW spiked with 0.05 µg L⁻¹ of selected OMPs.⁸ PQL is assessed as SD×10 which is in accordance with the rule that PQL is usually 3-5 times higher than MDL.⁸ Method repeatability is determined in a DCTW as the relative standard deviation of three measurements of different extracts. Instrument repeatability is determined as the relative standard deviation of three measurements in the same extract. Method and instrument repeatability are satisfactory (≤10 %) for all compounds and for both concentration levels (at 0.5 and 1.5 µg L⁻¹). Biases at both concentration levels were very satisfactory (≤8 %). They are determined based on the difference between measured calibration concentration and the expected calibration concentration (at 0.5 and 1.5 µg L⁻¹).

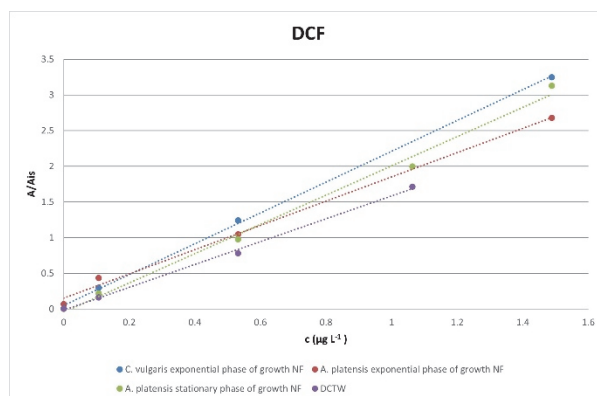
For calibrations in algal matrices, linearity was confirmed in range 0.1-1.5 µg L⁻¹ (Fig. S2). To cover variations in detector sensitivity, the peak area of each

analyte was divided by the peak area of the internal standard mecoprop (A/A_{is}). In applied linear range, no significant differences of A/A_{is} ratios were observed for the great majority of calibration points in DCTW and DCTW matrix containing algae (e.g. U.S. EPA Method 525.3 allows $\pm 30\%$ change in signal for quality control checks at mid- and high-calibration level points, while at the minimum level reporting $\pm 50\%$ of change from the expected value is allowed (U.S. EPA, 2012)¹⁰).



a) IB

b) CF



c) DCF

Fig. S2. Calibrations in DCTW and algal matrices; a) for IB; b) for CF and c) for DCF; DCTW- dechlorinated tap water; *Spirulina* NF – DCTW with *A. platensis* (*Spirulina*) addition (6.7×10^8 cells L^{-1}) and OMPs without filtration; *Spirulina* DF – DCTW with *A. platensis* (*Spirulina*) addition (6.7×10^8 cells L^{-1}) and OMPs double filtered through glass-fiber and afterwards $0.45 \mu m$ membrane filter; CHLORELLA NF – DCTW with *C. vulgaris* addition (3.5×10^8 cells L^{-1}) and OMPs without filtration; CHLORELLA DF – DCTW with *C. vulgaris* addition (3.5×10^8 cells L^{-1}) and OMPs filtered through a $0.45 \mu m$ membrane filter.

Thus, having the linear calibrations and assuming their validity for the samples before and after the treatment in each adsorption experiment, simple removal calculations were applied only from ratio of OMPs chromatographic signals and internal standard mecoprop (IS) (Eq. 1):

$$R = \frac{S_0 - S_i}{S_0} \cdot 100\% \quad (1)$$

whereas:

R denotes the relative removal of OMPs in percent

S_0 is the ratio of the peak area of the target ion of the OMP and the peak area of the internal standard target ion in the sample before the adsorption experiment (A_0/A_{IS0} , proportional to the initial concentration in the samples)

S_i is the ratio of the peak area of the target ion of the OMP and the peak area of the internal standard target ion in the sample after the adsorption experiment (A_i/A_{ISi} , proportional to the final concentration in the samples)

The samples of each matrix before treatment were filtered by the same procedure (GF and afterwards by MF) as the samples after PAC treatment.

Since the values before and after the adsorption experiment are measured independently from each other, the overall error of the relative removal can be calculated by the variance formula¹¹:

$$S_R = \sqrt{\left(\frac{\partial R}{\partial S_{S0}}\right)^2 \cdot S_{S0}^2 + \left(\frac{\partial R}{\partial S_{Si}}\right)^2 \cdot S_{Si}^2} \quad (2)$$

whereas:

S_R – overall error of the relative removal R

S_{S0} and S_{Si} are the measurement errors of S_0 and S_i . Combined uncertainty of OMPs analysis in DCTW matrix was used for their calculation. It was assessed based on adopted acceptance limit of 30% and the biases of recovery data up to 24% (see the sub-section *QC of GC/MS measurements*).

Solution of Eq. 2 results in Eq. 3:

$$s_R = \sqrt{\left(\frac{s_i}{s_0}\right)^2 \cdot s_{s0}^2 + \left(\frac{-1}{s_0}\right)^2 \cdot s_{si}^2} \quad (3)$$

DOC ANALYSIS

DOC in matrices was tracked using a TOC analyzer (liquiTOCII, Elementar, Germany). Method characterization and the results are shown in Table SIV and SV.

Table S-IV. DOC method characterization.

Linearity (mg L ⁻¹)	0.56–25
MDL (mg L ⁻¹)	0.25
PQL (mg L ⁻¹)	0.56
Trueness (%)	94–101
Precision at 1 mg L ⁻¹ (%)	13

Table S-V. Initial DOC values in matrices in the first and second experiment set.

Matrix	DOC, mgC/L		DOC difference between two sets, %	Impact of algae addition on DOC content in comparison to DCTW, %	
	I set	II set		I set	II set
DCTW blank	1.35	1.96	31	-	-
iiArth-e	1.29	1.81	29	4	-8
iiiArth-e	1.49	-	-	9	-
ivArth-e	2.23	-	-	39	-
vArth-e	1.65	-	-	18	-
DCTW blank	-	1.50	-	-	-
iiChlor-e	1.64	1.77	7	-	15
iiiChlor-e	1.97	1.78	-11	-	16
ivChlor-e	1.46	1.67	13	-	10
vChlor-e	1.54	1.93	20	-	22
DCTW blank	-	1.43	-	-	-
iiArth-s	1.70	1.69	-1	-	15
iiiArth-s	1.99	1.81	-10	-	21
ivArth-s	1.87	1.84	-2	-	22
vArth-s	2.29	1.96	-17	-	27
DCTW blank	-	1.84	-	-	-
iiChlor-s	1.58	1.49	-6	-	-23
iiiChlor-s	1.78	1.76	-1	-	-5
ivChlor-s	-	-	-	-	-
vChlor-s	1.77	1.95	9	-	6

Arth – *A. platensis*, Chlor – *C. vulgaris*, e – exponential growth phase, s – stationary growth phase. ii – non-chlorinated matrix with tAOM; iii – non-chlorinated matrix with dAOM; iv – pre-chlorinated matrix with tAOM; v – pre-chlorinated matrix with dAOM.; - not measured

RESULTS

Table S-VI. Comparison of the removal efficiency obtained in two set of experiments

Type of water matrix	Microalgae	Number of algae · 10 ⁸ (cells L ⁻¹)		Removal rates for AC1 ± error of the relative removal, %						Removal rates for AC2 ± error of the relative removal, %					
				IB		CF		DCF		IB		CF		DCF	
		I set	II set	I set	II set	I set	II set	I set	II set	I set	II set	I set	II set	I set	II set
i	without	-	-	47±14	77±6	77±7	93±2	93±2	90±3	36±17	69±8	76±8	92±3	99±0.3	85±4
	Arth-e	8	6	53±13	51±3	83±5	81±6	99±0.5	74±8	53±13	-	84±5	-	98±1	-
ii	Arth-s	4	5	66±9	-	89±4	-	87±4	-	41±16	47±14	81±6	89±4	62±11	63±11
	Chlor-e	2	3	47±14	-	89±4	-	88±4	-	NR	54±12	86±5	88±4	58±12	74±8
	Chlor-s	5	4	65±9	-	86±5	-	91±3	-	44±15	56±12	77±8	92±3	64±11	84±5
	Arth-e	8	-	54±12	-	83±6	-	89±3	-	16±23	-	73±9	-	92±2	-
iii	Arth-s	4	5	66±9	-	88±4	-	89±3	-	33±18	34±18	74±8	75±8	50±15	57±13
	Chlor-e	2	3	95±1	44±15	87±4	76±4	97±1	69±9	25±20	17±22	79±7	66±11	16±25	39±18
	Chlor-s	5	4	61±11	-	79±7	-	83±5	-	50±14	53±13	86±5	88±4	68±10	73±8
	Arth-e	8	-	61±10	-	83±6	-	97±1	-	37±17	-	79±7	-	90±3	-
iv	Arth-s	4	5	66±9	-	89±3	-	89±3	-	30±19	NR	73±9	19±26	44±17	30±21
	Chlor-e	2	3	70±8	63±10	90±3	88±8	91±3	78±7	57±12	NR	87±4	64±12	74±8	44±16
	Chlor-s	5	4	63±10	-	85±5	-	85±4	-	58±11	-	87±4	-	84±5	-
	Arth-e	8	-	55±12	-	76±8	-	89±3	-	39±16	-	78±7	-	79±6	-
v	Arth-s	4	5	60±11	-	87±4	-	82±5	-	39±17	39±17	79±7	80±7	54±14	60±12
	Chlor-e	2	3	63±10	-	88±4	-	88±4	-	14±23	28±19	78±7	79±7	43±17	54±14
	Chlor-s	5	4	66±9	-	66±11	-	86±4	-	36±17	33±18	65±11	81±6	47±16	74±8

Arth – *A. platensis*, Chlor – *C. vulgaris*, e – exponential growth phase, s – stationary growth phase. i – DCTW without algae; ii – non-chlorinated matrix with tAOM; iii – non-chlorinated matrix with dAOM; iv – pre-chlorinated matrix with tAOM; v – pre-chlorinated matrix with dAOM; - not repeated in second set experiments; NR – no removal

Based on the algal cell counts presented in Table SVI, it can be concluded that the deviation in algal abundance between first and second of experimental runs is not significant, as it falls within the known measurement uncertainty for algal quantification (20–30%)¹². A significant difference was observed between growth phases, as the difference in cell density between the exponential and stationary phases often exceeded 50%, which is well above the known 20–30% uncertainty of the hemocytometer counting method. While these differences reflect realistic physiological states of algae, they may have also contributed to the observed variability in OMP removal.

Quality control procedures (QC)

QC of experimental methodology

Adsorption test without algae - Experiment repeatability was checked by the analysis of five independent replicates of adsorption tests for AC2 in dechlorinated tap water (matrix (i), dose 5 mg L⁻¹, 2 h contact time, Figure S1). The relative standard deviation (RSD) of signal normalized for IS peak (A/A_{is}) was for IB, CF

and DCF 17, 2 and 0.8 %, respectively. Nine months later the triplicate of the same test gave RSD of 2 %, 1 % and 1 %, for IB, CF and DCF, respectively.

Adsorption test with algae – Repeatability was checked by three independent adsorption tests in matrix (ii), with AC2 dose 5 mg L⁻¹ and 2 h contact time (Figure S1). Both microalgae in stationary phase (4 and 5x10⁸ cells L⁻¹) were tested. RSD of signal normalized for IS peak (A/A_{IS}) in case of *C. vulgaris* was for IB 12 %, for CF 1 % and for DCF 0.1 %. In the case of *A. platensis* RSD of signal normalized for IS peak (A/A_{IS}) was ≤6 % for all OMPs.

Repeatability of adsorption tests in time - 14 experiments out of 32 were repeated after nine months (three for AC1 and 11 for AC2). Obtained results are shown in Table SVI. The values from the first set of adsorption test and the same tests repeated after 9 months were compared. All tests for AC1 in matrix containing algae showed differences less than 30 % for CF and for DCF, while for IB one showed 50 % difference, which is higher than expanded uncertainty of the analysis (38 %). For AC2, no tests with algae for DCF showed significant differences in removal rates, while for CF and IB, one and three tests had very low repeatability, respectively. A possible reason for this could be the fact that the matrices with algae are living systems and/or accuracy of preparing the solution varies as shown before in addition to the fact that accuracy of counting method of algal cells is between 20-30 %.¹² The applied criteria for declaring significant differences (> 30%) related to OMPs analysis is same as mentioned above.

QC of GC/MS measurements

Recovery test

An independent control standard check (OMPs c=0.5-0.7 µg L⁻¹) as the mid-calibration point was processed and analyzed exactly like a sample in laboratory routine work during one year. Single, same extract was repeatedly analyzed within one batch of measurements after each 10 samples (5-20 measurements of control standard within a batch). In total, data for 6 such batches were available during one year. Recoveries for all OMPs were between 78-124 %. During the analysis of the samples from the first set of experiments, this independent control standard check showed biases from expected values in the range 9-24 % with RSD of recovery values in the range of 1-2 % for all OMPs. Similarly, in the case of the second set of experiments, after nine months, biases from expected recovery values were in the range 7-20 % with RSD for recoveries of 3-4 % for all OMPs. Similar to the methodology provided in ISO/DIS 11352:2024¹³ standard, Annex C4, and based on the mentioned recovery values, combined uncertainty was calculated and obtained as 19 % for IB, 21 % for CF and 23 % for DCF.

Extraction and derivatization were monitored by check of internal standard mecoprop which was added into samples before extraction. For a total of 116 samples (32 experiments and 14 repeated, in total 12 batches of GC/MS analysis) RSD of target ion peak surface was 15 %, regardless of the water matrix used. In

the same time IS phenanthrene d-10 was used to assess the work of instrument without sample preparation and, only reflects gas-chromatography procedure. RSD of target ion peak surface was 19 %.

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