A new tool for the determination of humic substances in natural waters: pulsed voltammetry approach

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Abstract

Humic substances (HS) in natural waters can be determined with a new, simple and sensitive method based on their influence on the background current in a differential pulse - adsorptive cathodic stripping voltammogram. The proposed method, termed PB-HS (pulsed background - humic substances) is discussed in detail, including its application in natural samples from the Krka River estuary. The method was additionally compared with absorbance measurements as well as with the typical electrochemical HS quantification in natural waters based on HS complexation with molybdenum (Mo). A good correlation between methods was observed, with PB-HS showing slightly better sensitivity to humic compounds than classical spectrophotometry. Higher HS concentrations measured with the Mo-method may be due to the enhanced hydrophobicity reached at pH 2 that is required by the method. Advantages of the proposed PB-HS method, compared to existing voltammetric methods for HS quantification, are that it does not require any reagent addition (except buffer) and that it can be used at the natural pH of water as well as in a wide salinity range, which is crucial for its application in estuarine waters.

Keywords: humic substances, differential pulse adsorptive cathodic stripping voltammetry, seawater, estuary

1 Introduction

Humic substances (HS) are highly stable fractions of dissolved organic matter (DOM) and, as such, they contribute to long-term carbon storage in the deep ocean and play a major role in the global carbon geochemical cycle [1–3]. They are also strong metal-binding ligands and, thus, carriers of associated essential trace metals to the open ocean [4–6]. According to new findings, the significance of fluvial discharges in their global ocean input might be underestimated [4]. Understanding the land-to-ocean HS transfer is, therefore, an important subject concerning the global carbon budget, as well as for the ocean trace metal inventory. However, HS concentration in natural waters is not exactly known due to their complex structure and several analytical uncertainties [7]. Two operational fractions of aquatic HS can be distinguished from their solubility: (i) humic acid (HA), soluble in base, but insoluble in strong acid solutions and (ii) fulvic acid (FA), soluble throughout the pH range. Most humic substances are diverse, relatively small molecules between 100 and 2000 Da, which tend to form aggregate associations stabilized by hydrophobic interactions and hydrogen bonds, giving them macromolecular characteristics [8,9]. Their structure includes numerous carboxylic and phenolic groups in aromatic and alkyl moieties. Although HS are not true surfactants, they are described as surface-active substances (SAS) based on their effects on surface tension as a result of their amphiphilic nature [2].

Few practical electrochemical methods exist for HS quantification in natural waters. They are based on (i) surface-active organic compounds (i.e., SAS) interaction with the Hg drop electrode [10,11] and (ii) adsorptive properties of HS complexes with molybdenum (Mo) [12,13], iron (Fe) [7,14] or copper (Cu) [15]. The former methods use a modification of the capacitive current in phase sensitive alternating current (AC) voltammetry, pioneered by Ćosović and Vojvodić [16], or the suppression of the polarographic maximum of dissolved oxygen [17]. Since all types of SAS take part in the adsorption process at the electrode in a competitive manner, the methods are not substance specific, but quantify cumulative SAS presence in the sample. Different fractions of SAS can be further characterized with AC voltammetry by (i) comparing their DOC-specific surface activity with different model substances [18,19], (ii) estimating their hydrophobicity and acidity using o-nitrophenol as an electrochemical probe [20] or (iii) deriving kinetic adsorption constants at two different pH values and calibrating them against concentrations of various model substances [21]. Methods using adsorptive properties of HS complexes with metals are based on the measurement of a reduction peak preceded by the adsorptive collection of a given complex at the surface of the

Hg electrode in adsorptive cathodic stripping voltammetry (AdCSV). The Mo method operates at pH 2, while the Fe method uses a catalytic effect in the presence of bromate to obtain sufficient sensitivity.

The aim of the present work is to introduce a new simple and sensitive method for determining surface-active HS in seawater. The method is based on the HS influence on the background current in DP-AdCSV. The behaviour of model HS and some surfactants allows us to conclude that those surfactants do not interfere, so that the method reports only HS and not other SAS. Different methods are compared in samples from the Krka River estuary (Croatia).

2 Experimental part

2.1 Reagents and chemicals

Reagents and chemicals were prepared at room temperature (22 ± 1 °C) using ultrapure water (18.2 MΩ, Synthesis A10, Millipore, USA; referred hereafter as MQ) and stored in acid cleaned FEP (Fluorinated ethylene propylene), LDPE (Low density polyethylene) or PC (Polycarbonate) bottles. Hydrochloric acid (30% w/w HCl, *ROTIPURAN Supra*, Carl Roth) or nitric acid (69% w/w HNO_3 *ROTIPURAN Supra*, Carl Roth) were used for the preparation of reagents and acidification. Where needed, pH was maintained at 8.2 ± 0.1 (to simulate natural conditions in seawater) by the addition of a borate buffer containing 2.5 mol L^{-1} boric acid (H_3BO_3 *s.p.*, Sigma-Aldrich) and 0.4 mol L^{-1} NaOH. The final borate buffer concentration in solutions was 0.01 mol L^{-1}. pH was measured using a double junction pH electrode (Mettler-Toledo) calibrated against NIST (*National Institute of Standards and Technology*) traceable pH buffer solutions at 4.0, 7.0 and 10.0 (Reagecon). Molybdenum stock solutions were prepared by appropriate dilutions of an atomic absorption spectrometry standard solution (1 g L^{-1}, *TraceCERT*, Fluka) in HNO_3 (pH ~ 3).

Synthetic solutions for model experiments were prepared using organic-matter-free seawater (UVSW). UVSW was prepared by UV-digestion of filtered (0.45 µm cellulose-nitrate membrane filter) seawater for 24 h using a home-built system (250 W high-pressure mercury vapor lamp). The resulting DOC concentration after UV digestion was <7 µM. It was then purified using a MnO_2 suspension [22] for at least 12 h at room temperature before triple filtration through pre-cleaned 0.45 and 0.2 µm filters.

Standard samples of humic and fulvic acids were obtained from the International Humic Substances Society (IHSS) and Sigma-Aldrich: IHSS Suwannee River fulvic acid (SRFA;

3S101F), IHSS Suwannee River humic acid (SRHA; 3S101H) and Sigma-Aldrich humic acid (HA; 600–1000 Da, CAS: 1415-93-6). Elemental Compositions and Stable Isotopic Ratios of IHSS Samples are given on the IHSS official website [23]. The fulvic acid stock solution was prepared by dissolution in MQ water, whereas humic acid stock solutions were prepared by dissolution in 1 mmol L^{-1} NaOH. Prepared solutions were stored at 4 °C. Additionally, humic substances isolated from the St. Lawrence estuary according to IHSS protocol were used: HA (SLE-HA), FA (SLE-FA), hydrophobic organic matter (SLE-Hphobe) and hydrophilic organic matter (SLE-Hphile). SLE-solutions were a gift from Dr. Jean-Pierre Gagné from the "Institut des sciences de la mer de Rimouski, Université du Québec à Rimouski, Québec, Canada" through Dr. Cédric Garnier from the University of Toulon (France). Triton-X-100, dextran (produced by *Leuconostoc mesenteroides*, 2000 kDa), ι-carrageenan (type V, produced by *Eucheuma spinosa*), human serum albumin (HSA, fatty acid free), chitosan oligosaccharide lactate (5000 Da) and xanthan gum (from *Xanthomonas campestris*, 2000 kDa), all purchased from Sigma-Aldrich, were used as additional model surface-active substances.

2.2 Sampling

Water samples were collected in the stratified Krka River estuary (Croatia) at Station M (43° 44′ 07.92 N, 15° 52′ 39.61 E; in front of Šibenik town) (**Figure S1** in the Supplementary Material) during five sampling campaigns: July and December 2017, July 2018, February and July 2019. In all campaigns, samples were collected from six depths covering a range of salinities from brackish to pure seawater using a van Dorn-type horizontal sampler. Samples were filtered within 2 h of collection at the onshore laboratory using pre-cleaned (with 1% v/v trace metal-clean HNO_3) 0.45 μm cellulose-nitrate membrane filters (47 mm in diameter; Sartorius) under nitrogen pressure (~ 1 bar), using a Sartorius polycarbonate filter holder (model 16511).

2.3 Measurements and instrumentation

2.3.1 Voltammetric measurements

Voltammetric measurements were carried out in a three-electrode cell (663 VA Stand, Metrohm) controlled by a Metrohm-Autolab (Eco Chemie) potentiostat/galvanostat (μAutolab Type III) in conjunction with the IME663 module (Metrohm-Autolab) interfaced with the GPES (General Purpose Electrochemical System) v.4.9 software (Eco Chemie). The three-electrode system consisted of (i) a hanging mercury drop (HMDE; MME, Metrohm) (drop size: 0.40 mm^2 unless indicated otherwise), serving as the working electrode, (ii) a glassy carbon rod, serving as the counter electrode and (iii) an Ag|AgCl|sat. NaCl electrode (model 6.0728.120+6.1245.010, Metrohm), containing purified UVSW as the outer filling solution in the bridge (used in all measurements), serving as the reference electrode. A home-made quartz or PFA (Metrohm) cells were used. In order to remove reactive dissolved oxygen, the sample (10–12 mL) was purged with nitrogen for at least 5 minutes prior to the first measurement and for 15–30 s between separate measurements in the same sample. During the measurement, a nitrogen blanket was maintained over the sample. The solution was stirred during the nitrogen purging and during the deposition step, using the rotating stirrer at 3000 rpm.

Pulsed-Background HS method

This work proposes a new method for the quantification of HS in the sample, named ‘Pulsed-Background HS’ (PB-HS) method. Argumentation of the proposed method is thoroughly described later in the *Results and discussion* section. Briefly, the method is based on the elevation of the background current as a function of the concentration of surface-active HS adsorbed onto the electrode. The HS were quantified in terms of equivalent SRHA reference material. Final instrumental settings used for measurement in the samples are given in **Table 1**. Based on the presented results in this work, we suggest the following analytical protocol for the determination of HS in natural waters:

1. put 10 mL of filtered sample in the voltammetric cell,
2. buffer it up to 0.01 mol L^{-1} borate/ammonia buffer (the final concentration in the cell)
3. perform the baseline scan using the instrumental settings given in **Table 1** and a deposition time of 2 s,
4. perform the HS measurement (i.e. analytical scan) using instrumental settings given in **Table 1** and a deposition time of 60 s,
5. calculate the differential current (labelled ΔI) as the average, in the -0.75 to -0.80 V potential range of the differences between the currents of the analytical and the baseline scans,
6. determine HA_{eq} (the equivalent concentration of HA, as indicated by the subscript “eq”) with the standard addition method by repeating steps 2–5 for each addition of the reference material (SRHA preferably) and considering that ΔI is proportional to HA_{eq}

concentration.

It should be emphasized that the complete removal of oxygen from the electrochemical cell and the test solution is very important because its presence can interfere with measurements by increasing the baseline current. Furthermore, the mercury pool inside of the holder and the capillary should be free of mercury oxides, which are known to prevent getting a proper voltammograms at low current ranges.

The calculation of LOD and LOQ of the new method was based on the standard addition plot with equation provided in page 124 of Miller and Miller [24]. LOD and LOQ were computed for 1 min of accumulation time in UVSW with additions of IHSS SRHA up to 0.6 mg/L. The following values were estimated: LOD = 0.020 mg L^{-1} and LOQ = 0.066 mg L^{-1} of IHSS SRHA as a model organic compound. Obviously, the LOD and LOQ could be reduced by increasing the accumulation time.

Molybdenum-HS method

HS concentrations were determined by acidifying the sample to pH ~ 2 in the voltametric cell and then adding Mo(VI) up to 100 nmol L^{-1}. This was followed by a 60 s deposition at a potential of -0.20 V [12,13]. The reduction of adsorbed Mo-HS complexes in the AdCSV stripping step resulted in a peak at ~ -0.50 V (**Figure S2**). The Mo-HS peak height was expressed as equivalent of IHSS SRHA (Mo-HA_{eq}), and the quantification was performed by using the standard addition method.

Table 1.

2.3.2 Dissolved organic carbon and absorbance measurements

Additional measurements of dissolved organic carbon (DOC) and absorbance were performed in the studied estuarine samples. Measurements are described in detail in Marcinek et al., 2020 [25]. Briefly, DOC concentration was determined by high temperature catalytic oxidation using the Shimadzu On-line TOC-VCSH carbon analyser. UV/Vis absorbance was measured using a JASCO V-550 or Specord 200 Plus (Analytik Jena GmbH) spectrophotometer with a 10 cm Suprasil quartz cuvette. Absorbance at 254 nm (A_{254}) was used as representative of the chromophoric organic matter pool and expressed as the absorption coefficient (a_{254}) in Napierian units.

3 Results and discussion

During the measurement of copper-binding HS according to the method proposed by Whitby and van den Berg, 2015 [15], we noticed the significant increase of the whole background current after the addition of the HS standard to the sample (**Figure 1**). In AC voltammetry, the adsorption of SAS on the electrode surface at the potential of the electrocapillary maximum of the mercury electrode (~ -0.6 V, at given conditions) results in the decrease of the capacitive current relative to a blank electrolyte [26]. However, using pulsed techniques (e.g. differential pulse or square wave voltammetry) after a deposition (adsorption) step (whose combination can be labelled as DP-AdCSV), the elevation of the whole background current as a function of HS concentration was observed, as shown in **Figure 1**. It was obvious that this was caused by SRHA adsorption on the surface of the Hg drop during the deposition step, as it was found that the increase is proportional to the deposition time (data not shown). With a short deposition time (2 s), there was a small increase of the baseline current relative to the UVSW blank (with no HS), suggesting that the current increase was proportional to the amount of adsorbed HS at the electrode surface during the deposition step. It seemed worth exploring whether the specific behaviour observed for SRHA could be used to determine SAS (HS) concentration in natural waters, and possibly even discriminate between various SAS compounds, as demonstrated and discussed in the following sections.

Figure 1.

3.1 Influence of various model SAS on background currents in DP-AdCSV

The influence of various HS standards on the background current was tested, including Suwannee River HA (SRHA) and FA (SRFA) obtained from IHSS, as well the HA (SLE-HA), FA (SLE-FA), more hydrophobic organic matter (SLE-Hphobe) and more hydrophilic organic matter (SLE-Hphile), isolated from the St. Lawrence estuary. An increase in the background current as a function of substance concentration and deposition time was observed for all tested HS. The differential current (ΔI) in the -0.75 to -0.80 V potential range (average current in this range) relative to the baseline scan (2 s deposition time) was chosen as a parameter for the quantification of these substances. The signal around the mentioned potentials is well resolved and without interference, such as for example the formation of a thiol peak at ~ -0.55 V or possible Fe interference at a potential of ~ -0.60 V in natural samples [7], as can be seen in **Figure 1**. The peak at -0.20 V (**Figure 1**) is a Cu-HA complex formed after the addition of 50 nmol L^{-1} Cu to the sample to ensure that all Cu-binding sites on the added HS are saturated. This peak is observed in the quantification of Cu-binding HS in seawater following the method proposed by Whitby and van den Berg, 2015 [15]. The same experiments can, thus, be used for the simultaneous determination of HS (with the new method) and the Cu-HS complex.

Figure 2.

The PB-HS method showed different sensitivities to various types of riverine and estuarine HS, suggesting an impact of the different nature of the different HS. Calibration lines of tested HS standards, at a fixed t_{dep} = 60 s, are given in **Figure 2**. The highest sensitivities were observed for the IHSS reference materials, with slopes of 4.2 and 2.9 μA g^{-1} L for SRHA and SRFA, respectively. HS isolated from the St. Lawrence estuary showed lower sensitivities than IHSS standards, with slopes of 2.3 and 0.6 μA g^{-1} L for SLE-HA and SLE-FA, respectively. The sensitivity of the hydrophilic fraction (SLE-Hphile) was in between those observed for SLE-HA and SLE-FA with a slope of 1.1 μA g^{-1} L, while the hydrophobic organic matter (SLE-Hphobe) had the lowest sensitivity with a slope of 0.3 μA g^{-1} L.

Our measurements were performed using the adsorption potential of 0 V. However, the increase of the background current (> 60%) from E_{dep} 0 to -0.50 V (**Figure S3**) and the fall of the intensity after the potential of zero charge on the mercury electrode under typical seawater conditions, probably indicate that a combination of hydrophobicity and electrostatic attraction yield the observed change of trend in SRHA adsorption as a function of the charge of the HMDE (which is a function of E_{dep}).

The new PB-HS method (based on DP-AdCSV) was tested for other model substances as well (**Figure S4)**. Triton-X-100 was chosen because it is a typical non-ionic surfactant known for its strong adsorption at the mercury electrode and a model compound for the quantification of SAS in natural waters, representing the most reactive part of SAS when using AC (out-of-phase) voltammetry [16,27]. Dextran and carrageenan were chosen as models of microbially and algal derived polysaccharides, respectively [28–30], whereas human serum albumin (HSA) was chosen as a model of protein substances [31,32]. Other tested model substances that are frequently found in seawater were: chitosan oligosaccharide lactate, a cationic biopolymer which is a major component of N-bearing polymeric organic material in seawater [33] and the hydrophilic microbial polysaccharide xanthan gum which is a precursor of transparent exopolymer particles in seawater [34].

Unlike HS standards, the addition of Triton-X-100, dextran, HSA and xanthan gum in the UVSW had no significant effect on the background current (**Figure S4A-D**). A small peak observed around -0.50 V after 60 s of deposition at 0.0 V is likely due to the reduction of Hg-sulphur species formed at the electrode surface [35–37]. However, this peak is not influencing performance of our method. The addition of carrageenan caused the occurrence of small peaks in the potential range between -0.40 V and -0.65 V (**Figure S4E**), uncommon for natural oxic samples, but not the elevation of the whole background current as was observed for HS. Carrageenan of ι-type has a sulphur content of 7%, so these peaks might be a result of the Hg-species reduction processes due to the interaction of the electrode with its built-in S-groups [38]. A similar reduction peak was found in aggregates of diatom cultures upon incubation with sulphide in anoxic conditions, which was ascribed to the presence of organosulfur species [38].

The combined effect of Triton-X-100, carrageenan and dextran (added at higher concentrations than in the previous experiments) on the SRHA response in UVSW was also tested (**Figure S5**), showing the same results as when these substances were tested on their own. For carrageenan, the same reduction peaks were observed. The reduction peaks between -0.50 and -0.70 V appeared after the addition of dextran, as well. Essentially, peaks that appeared after the addition of dextran and carrageenan had no effect on the background currents at potentials more negative than -0.75 V. Therefore, as DP-AdCSV is not sensitive to all SAS, it can potentially be used to resolve the humic fraction from the mixed pool of SAS in natural samples which is a great advantage of the proposed PB-HS method.

An open question is the elucidation of the physicochemical mechanism behind the background current increase. As for now, we very briefly discuss two justifications. At first, we would be inclined to believe that this phenomenon is connected to some charging current increase (in absolute value, after applying the pulse in DPV technique) for increased HS adsorbed on the electrode (i.e. a capacitance increase), but AC voltammetry finds that the capacitance decreases with increased HS adsorption, so we cannot clearly endorse this mechanism. However, another possible explanation is a pseudocapacitive behaviour of HS. Namely, humic substances contain abundant redox-active oxygen-containing functional groups such as phenol, carbonyl, quinones or carboxyl, which are known to endow pseudocapacitive behaviour to HS [39–41]. HS are large molecules with numerous active sites that exhibit different redox potentials across a wide potential range [42]. Our control measurements with cyclic voltammetry showed no reduction/oxidation peaks, but there was a clear increase in cathodic (reduction) background current, which increased with the addition of HS and with increasing adsorption time. Hence, we hypothesise that the increase of background current observed in our measurements might be (totally or just partially) the result of the (irreversible) reduction of the redox active sites of the adsorbed HS. The resulting current is usually termed as “pseudocapacitive current” (faradaic non-diffusion limited) [40]. The active sites of adsorbed HS on the surface of the Hg electrode are located at different distances from the electrode surface, posing the need for larger reduction overpotentials for some of them, and resulting in the global increase of the (background) current along our scanned potential range. In this work, we primarily focused on the empirical effect that we observed and its application for the determination of HS in natural waters. Further work should elucidate the mechanism behind the observed phenomena.

3.2 Experimental verification in natural samples

The new PB-HS method was tested in samples from the vertical profile of the Krka River estuary in two contrasting seasons, winter and summer, during five sampling campaigns (**Figure 3**). Quantification of the unknown HS concentration was performed using the standard addition method. IHSS SRHA was used as a model reference material, and thus the results are expressed as equivalents of SRHA in mg L^{-1} (HA_{eq}).

Before the measurements in real estuarine samples, we tested the performance of the PB-HS method at different salinities and pH. These experiments showed that the sensitivity of the method is independent from pH and salinity in the range 10 to 38 (**Figure S6**). There is a possibility of a different response at lower salinities than those explored here. However, considering that the concentration of HS is determined by the standard addition method, a change of sensitivity would not be important. In any case, these experiments showed that the method is valid in a wide salinity and pH range, which is crucial for its application in estuarine waters.

Figure 3.

The distributions of HA_{eq} and DOC-normalized HA_{eq} in the salinity gradient for all sampling campaigns presented in **Figure 3** show that HA_{eq}, as well as the abundance of HS in the DOM pool decreased when salinity increased. The lowest salinity (< 15) was observed in surface waters during the winter campaigns. DOM in these samples contained the highest percentage of HS indicated by the highest HA_{eq}/DOC ratio. The DOM content in surface waters in the winter period was low and mainly consisted of terrestrial humic substances, as already shown earlier in a study focussed on the optical properties of DOM in this area [25].

The PB-HS method was additionally compared with absorbance measurements in the same samples (**Figure 4)** where a relationship between HA_{eq} and a_{254} is revealed. The absorption coefficient at 245 nm (a_{254}) can be used as surrogate parameter to follow the presence of HS in marine studies, based on the assumption that light absorption is mainly caused by aromatic structures which are major constituents of HS [43,44]. The points in the circled area of the figure correspond to winter samples in surface water (salinity < 15). The deviation of these points from the general trend could arise from the PB-HS method being slightly more sensitive towards humic compounds than classical spectrophotometry and because a_{254} is sensitive to other types of organic matter as well, such as freshly produced biogenic material that are not present in these winter samples [25]. The plotted relationship tends to have a small positive X-intercept which could be due to the increase of the absorbance that occurs in seawater in the low spectral range as a result of salinity itself.

Figure 4

The results of the PB-HA method were also compared to those of Mo-HS method (**Figure 5**), often used for electrochemical HS quantification in natural waters [45–48]. A very good correlation between the two methods was obtained. However, ~ 3× higher HA_{eq} were obtained using the Mo-method than with the PB-HS one. A previous intercomparison of the Mo-method with absorbance measurements [49], as well with the Fe-method [7], found slightly higher HS concentrations in seawater with the Mo-method, although not as high as with the PB-HS method. Laglera et al., 2007 [7] assumed that it is caused by a gradual change in the oxidation state of Mo over the scanned potential range. Moreover, protonation of ionic groups in HS can enhance their hydrophobic nature below pH 6, facilitating their adsorption on the Hg electrode [50,51], which might be a reason (not exclusive with others) for higher HA_{eq} observed with the Mo-HS method at pH 2 than with the PB-HS and Fe-HS methods at pH 8.2. For example, in samples rich in organic matter of anionic character, the adsorption effect was found to be up to 45% higher in acidic than in neutral medium [51,52]. The observed difference clearly indicates that these two methods determine different fractions of the DOM pool and should be considered as complementary methods. In any case, it would be worth to further investigate the reasons for the divergence in absolute concentrations of HA determined by these two methods.

Figure 5

4 Conclusion

This work proposes a new method to resolve the humic fraction from the mixed pool of surface-active substances in natural waters based on their influence on the background current in a differential pulse - adsorptive cathodic stripping voltammetry. The method is fast and simple, it does not require any addition of reagents beside buffer and is suitable for measurements in a wide salinity and pH range, which is a great advantage compared to existing voltammetric methods for HS quantification. The method was successfully used for the determination and monitoring of the distribution of HS in the Krka River estuary showing a decrease of HS abundance in the DOM pool with increasing salinity. The still open question about the electrochemical mechanism behind the increase in the background current is planned to be tackled in future work.

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References

[1] P.M. Medeiros, M. Seidel, J. Niggemann, R.G.M. Spencer, P.J. Hernes, P.L. Yager, W.L. Miller, T. Dittmar, D.A. Hansell, A novel molecular approach for tracing terrigenous dissolved organic matter into the deep ocean, Global Biogeochem. Cycles. 30 (2016) 689–699. https://doi.org/10.1002/2015GB005320.

[2] M. Klavins, O. Purmalis, Humic substances as surfactants, Environ. Chem. Lett. 8 (2010) 349–354. https://doi.org/10.1007/s10311-009-0232-z.

[3] N. Jiao, G.J. Herndl, D.A. Hansell, R. Benner, G. Kattner, S.W. Wilhelm, D.L.

Kirchman, M.G. Weinbauer, T. Luo, F. Chen, F. Azam, Microbial production of recalcitrant dissolved organic matter: long-term carbon storage in the global ocean, Nat. Rev. Microbiol. 8 (2010) 593–599. https://doi.org/10.1038/nrmicro2386.

[4] F.L.L. Muller, Exploring the Potential Role of Terrestrially Derived Humic Substances in the Marine Biogeochemistry of Iron, Front. Earth Sci. 6 (2018). https://doi.org/10.3389/feart.2018.00159.

[5] R.M. Bundy, H.A.N. Abdulla, P.G. Hatcher, D. V. Biller, K.N. Buck, K.A. Barbeau, Iron-binding ligands and humic substances in the San Francisco Bay estuary and estuarine-influenced shelf regions of coastal California, Mar. Chem. 173 (2015) 183–194. https://doi.org/10.1016/j.marchem.2014.11.005.

[6] R. Yang, C.M.G. van den Berg, Metal Complexation by Humic Substances in Seawater, Environ. Sci. Technol. 43 (2009) 7192–7197. https://doi.org/10.1021/es900173w.

[7] L.M. Laglera, G. Battaglia, C.M.G. van den Berg, Determination of humic substances in natural waters by cathodic stripping voltammetry of their complexes with iron, Anal. Chim. Acta. 599 (2007) 58–66. https://doi.org/10.1016/j.aca.2007.07.059.

[8] J. Balch, C. Guéguen, Effects of molecular weight on the diffusion coefficient of aquatic dissolved organic matter and humic substances, Chemosphere. 119 (2015) 498–503. https://doi.org/10.1016/j.chemosphere.2014.07.013.

[9] J.A. Leenheer, J.P. Croué, Characterizing aquatic dissolved organic matter, Environ. Sci. Technol. 31 (2003) 18a-26a.

[10] B. Ćosović, V. Vojvodić, Direct determination of surface active substances in natural waters, Mar. Chem. 22 (1987) 363–373.

[11] B. Ćosović, P. Orlović Leko, Z. Kozarac, Rainwater dissolved organic carbon: Characterization of surface active substances by electrochemical method, Electroanalysis. 19 (2007) 2077–2084. https://doi.org/10.1002/elan.200703907.

[12] F. Quentel, C. Elleouet, Square-wave voltammetry of molybdenum-fulvic acid complex, Electroanalysis. 13 (2001) 1030–1035. https://doi.org/10.1002/1521-4109(200108)13:12<1030::AID-ELAN1030>3.0.CO;2-6.

[13] F. Quentel, M. Filella, Quantification of refractory organic substances in freshwaters: Further insight into the response of the voltammetric method, Anal. Bioanal. Chem. 392 (2008) 1225–1230. https://doi.org/10.1007/s00216-008-2366-4.

[14] C. Sukekava, J. Downes, H.A. Slagter, L.J.A. Gerringa, L.M. Laglera, Determination of the contribution of humic substances to iron complexation in seawater by catalytic cathodic stripping voltammetry, Talanta. 189 (2018) 359–364. https://doi.org/10.1016/j.talanta.2018.07.021.

[15] H. Whitby, C.M.G. van den Berg, Evidence for copper-binding humic substances in seawater, Mar. Chem. 173 (2015) 282–290. https://doi.org/10.1016/j.marchem.2014.09.011.

[16] B. Ćosović, V. Vojvodić, The application of ac polarography to the determination of surface- active substances in seawater, Limnol. Oceanogr. 27 (1982) 361–369. https://doi.org/10.4319/lo.1982.27.2.0361.

[17] A. Cominoli, J. Buffle, W. Haerdi, Voltammetric study of humic and fulvic substances, J. Electroanal. Chem. Interfacial Electrochem. 110 (1980) 259–275.

https://doi.org/10.1016/S0022-0728(80)80378-0.

[18] B. Ćosović, V. Vojvodić, Voltammetric analysis of surface active substances in natural seawater, Electroanalysis. 10 (1998) 429–434. https://doi.org/10.1002/(SICI)1521-4109(199805)10:6<429::AID-ELAN429>3.0.CO;2-7.

[19] I. Ciglenečki, P. Orlović-Leko, K. Vidović, N. Simonović, M. Marguš, J. Dautović, S. Mateša, I. Galić, The possibilities of voltammetry in the study reactivity of dissolved organic carbon (DOC) in natural waters, J. Solid State Electrochem. (2023). https://doi.org/10.1007/s10008-023-05423-y.

[20] B. Gašparović, B. Ćosović, Surface-active properties of organic matter in the North Adriatic Sea, Estuar. Coast. Shelf Sci. 58 (2003) 555–566. https://doi.org/10.1016/S0272-7714(03)00133-1.

[21] M. Cuscov, F.L.L. Muller, Differentiating humic and algal surface active substances in coastal waters by their pH-dependent adsorption behaviour, Mar. Chem. 174 (2015) 35–45. https://doi.org/10.1016/j.marchem.2015.05.002.

[22] C.M.G. van den Berg, Chemical speciation of iron in seawater by cathodic stripping voltammetry with dihydroxynaphthalene, Anal. Chem. 78 (2006) 156–163. https://doi.org/10.1021/ac051441+.

[23] IHSS, International Humic Substances Society, (2022). https://humic-substances.org/elemental-compositions-and-stable-isotopic-ratios-of-ihss-samples/.

[24] J.N. Miller, J.C. Miller, Statistics and chemometrics for analytical chemistry, 6th ed., Pearson Education Limited, Harlow, 2010.

[25] S. Marcinek, C. Santinelli, A.M. Cindrić, V. Evangelista, M. Gonnelli, N. Layglon, S. Mounier, V. Lenoble, D. Omanović, Dissolved organic matter dynamics in the pristine Krka River estuary (Croatia), Mar. Chem. 225 (2020). https://doi.org/10.1016/j.marchem.2020.103848.

[26] B. Ćosović, Z. Kozarac, S. Frka, V. Vojvodić, Electrochemical adsorption study of natural organic matter in marine and freshwater systems. A plea for use of mercury for scientific purposes, Electroanalysis. 22 (2010) 1994–2000. https://doi.org/10.1002/elan.201000041.

[27] I. Ciglenečki, I. Vilibić, J. Dautović, V. Vojvodić, B. Ćosović, P. Zemunik, N. Dunić, H. Mihanović, Dissolved organic carbon and surface active substances in the northern Adriatic Sea: Long-term trends, variability and drivers, Sci. Total Environ. 730 (2020). https://doi.org/10.1016/j.scitotenv.2020.139104.

[28] M. Plavšić, B. Ćosović, Adsorption properties of different polysaccharides on mercury in sodium chloride solutions, Electroanalysis. 12 (2000) 895–900. https://doi.org/10.1002/1521-4109(200008)12:12<895::AID-ELAN895>3.0.CO;2-W.

[29] S.D.S. Strmečki, M. Plavšić, An ex situ electrocatalytic analysis of κ-, ι-, and λ-carrageenan on mercury electrode in seawater, J. Electroanal. Chem. 712 (2014) 1–7. https://doi.org/10.1016/j.jelechem.2013.10.023.

[30] M. Plavšić, S. Strmečki, Carbohydrate polymers as constituents of exopolymer substances in seawater, their complexing properties towards copper ions, surface and catalytic activity determined by electrochemical methods, Carbohydr. Polym. 135 (2016) 48–56. https://doi.org/10.1016/j.carbpol.2015.08.071.

[31] S. Strmečki, M. Plavšić, B. Ćosović, Constant current chronopotentiometric stripping

analysis of ‘N-Catalyst’ in sodium chloride solution and seawater, Electroanalysis. 22 (2010) 91–98. https://doi.org/10.1002/elan.200900233.

[32] S. Strmečki, J. Dautović, M. Plavšić, Constant current chronopotentiometric stripping characterisation of organic matter in seawater from the northern Adriatic, Croatia, Environ. Chem. 11 (2014) 158–166. https://doi.org/10.1071/EN13122.

[33] S. Strmečki, L. Pereža, Electrochemistry of chitosan amino-glycan and BSA protein mixture under seawater conditions, J. Electroanal. Chem. 898 (2021). https://doi.org/10.1016/j.jelechem.2021.115630.

[34] X. Li, L. Skillman, D. Li, W.P. Ela, Comparison of Alcian blue and total carbohydrate assays for quantitation of transparent exopolymer particles (TEP) in biofouling studies, Water Res. 133 (2018) 60–68. https://doi.org/10.1016/j.watres.2017.12.021.

[35] E. Bura-Nakić, G.R. Helz, I. Ciglenečki, B. Ćosović, Reduced sulfur species in a stratified seawater lake (Rogoznica Lake, Croatia); seasonal variations and argument for organic carriers of reactive sulfur, Geochim. Cosmochim. Acta. 73 (2009) 3738–3751. https://doi.org/10.1016/j.gca.2009.03.037.

[36] P.-J. Superville, I. Pižeta, D. Omanović, G. Billon, Identification and on-line monitoring of reduced sulphur species (RSS) by voltammetry in oxic waters, Talanta. 112 (2013) 55–62. https://doi.org/10.1016/j.talanta.2013.03.045.

[37] A. Cvitešić Kušan, S. Frka, I. Ciglenečki, Electrochemical evidence of non-volatile reduced sulfur species in water-soluble fraction of fine marine aerosols, Atmosphere (Basel). 10 (2019) 674. https://doi.org/10.3390/atmos10110674.

[38] I. Ciglenečki, M. Plavšić, V. Vojvodić, B. Ćosović, M. Pepi, F. Baldi, Mucopolysaccharide transformation by sulfide in diatom cultures and natural mucilage, Mar. Ecol. Prog. Ser. 263 (2003) 17–27. https://doi.org/10.3354/meps263017.

[39] F.N. Ajjan, D. Mecerreyes, O. Inganäs, Enhancing energy storage devices with biomacromolecules in hybrid electrodes, Biotechnol. J. 14 (2019). https://doi.org/10.1002/biot.201900062.

[40] C. Wang, T. Cheng, D. Zhang, X. Pan, Electrochemical properties of humic acid and its novel applications: A tip of the iceberg, Sci. Total Environ. 863 (2023) 160755. https://doi.org/10.1016/j.scitotenv.2022.160755.

[41] K. Wasiński, M. Walkowiak, G. Lota, Humic acids as pseudocapacitive electrolyte additive for electrochemical double layer capacitors, J. Power Sources. 255 (2014) 230–234. https://doi.org/10.1016/j.jpowsour.2013.12.140.

[42] J.T. Nurmi, P.G. Tratnyek, Electrochemical properties of natural organic matter (NOM), fractions of NOM, and model biogeochemical electron shuttles, Environ. Sci. Technol. 36 (2002) 617–624. https://doi.org/10.1021/es0110731.

[43] D. Hansell, C.A. Carlson, Marine dissolved organic matter and the carbon cycle, Oceanography. 14 (2001) 41–49. https://doi.org/10.5670/oceanog.2001.05.

[44] M. Filella, Quantifying ‘humics’ in freshwaters: purpose and methods, Chem. Ecol. 26 (2010) 177–186. https://doi.org/10.1080/02757540.2010.494159.

[45] R. Riso, M. Mastin, A. Aschehoug, R. Davy, J. Devesa, A. Laës-Huon, M. Waeles, G. Dulaquais, Distribution, speciation and composition of humic substances in a macro-tidal temperate estuary, Estuar. Coast. Shelf Sci. 255 (2021) 107360.

https://doi.org/10.1016/j.ecss.2021.107360.

[46] G. Dulaquais, M. Waeles, J. Breitenstein, J. Knoery, R. Riso, Links between size fractionation, chemical speciation of dissolved copper and chemical speciation of dissolved organic matter in the Loire estuary, Environ. Chem. 17 (2020) 385. https://doi.org/10.1071/EN19137.

[47] B. Pernet-Coudrier, M. Waeles, M. Filella, F. Quentel, R.D. Riso, Simple and simultaneous determination of glutathione, thioacetamide and refractory organic matter in natural waters by DP-CSV, Sci. Total Environ. 463–464 (2013) 997–1005. https://doi.org/10.1016/j.scitotenv.2013.06.053.

[48] Z. Gao, C. Guéguen, Distribution of thiol, humic substances and colored dissolved organic matter during the 2015 Canadian Arctic GEOTRACES cruises, Mar. Chem. 203 (2018) 1–9. https://doi.org/10.1016/j.marchem.2018.04.001.

[49] F. Quentel, C. Madec, A.L. Bihan, J. Courtot-coupez, Determination des substances humiques en milieu marin par redissolution cathodique a l’electrode a coutte pendante de mercure, Anal. Lett. 19 (1986) 325–344.

[50] M. Terashima, M. Fukushima, S. Tanaka, Influence of pH on the surface activity of humic acid: Micelle-like aggregate formation and interfacial adsorption, Colloids Surfaces A Physicochem. Eng. Asp. 247 (2004) 77–83. https://doi.org/10.1016/j.colsurfa.2004.08.028.

[51] B. Ćosović, P.O. Leko, Z. Kozarac, Rainwater dissolved organic carbon: Characterization of surface active substances by electrochemical method, Electroanalysis. 19 (2007) 2077–2084. https://doi.org/10.1002/elan.200703907.

[52] P. Orlović-Leko, K. Vidović, M. Plavšić, I. Ciglenečki, I. Šimunić, T. Minkina, Voltammetry as a tool for rough and rapid characterization of dissolved organic matter in the drainage water of hydroameliorated agricultural areas in Croatia, J. Solid State Electrochem. 20 (2016) 3097–3105. https://doi.org/10.1007/s10008-016-3245-0.

Table 1. Instrumental settings used for the voltammetric measurements of HA_{eq}.

Parameters	**PB-HS (natural pH)**	**Mo-HS (pH ~ 2)**
Deposition potential (V)	0	-0.2
Deposition duration (s)	2 or 60	60
Equilibration time (s)	5	5
Modulation time (s)	0.04	0.04
Interval time (s)	0.10	0.10
Potential scan (V)	-0.05 to -0.9	-0.05 to -0.9
Step potential (V)	0.002	0.002
Modulation amplitude (V)	0.04	0.04

Figure Captions

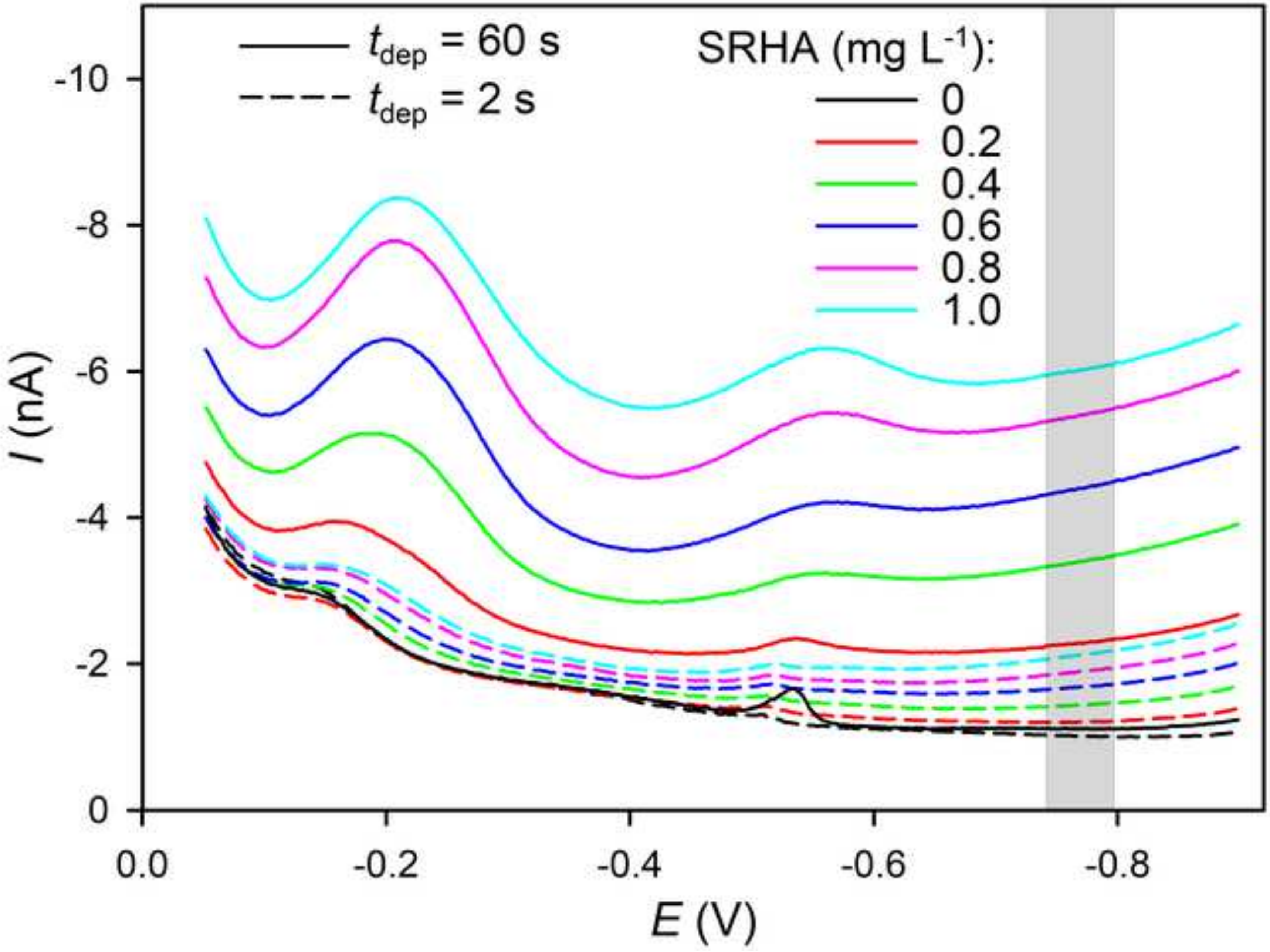
Figure 1. DP-AdCSV voltammograms obtained in UVSW with added 50 nmol L^{-1} Cu at increasing concentrations of SRHA. Electrochemical parameters are given in **Table 1**. The shaded area represents the potential region where the averaged differential current (difference between the currents of the analytical scan - solid lines and those of the baseline scan - dashed lines) was determined.

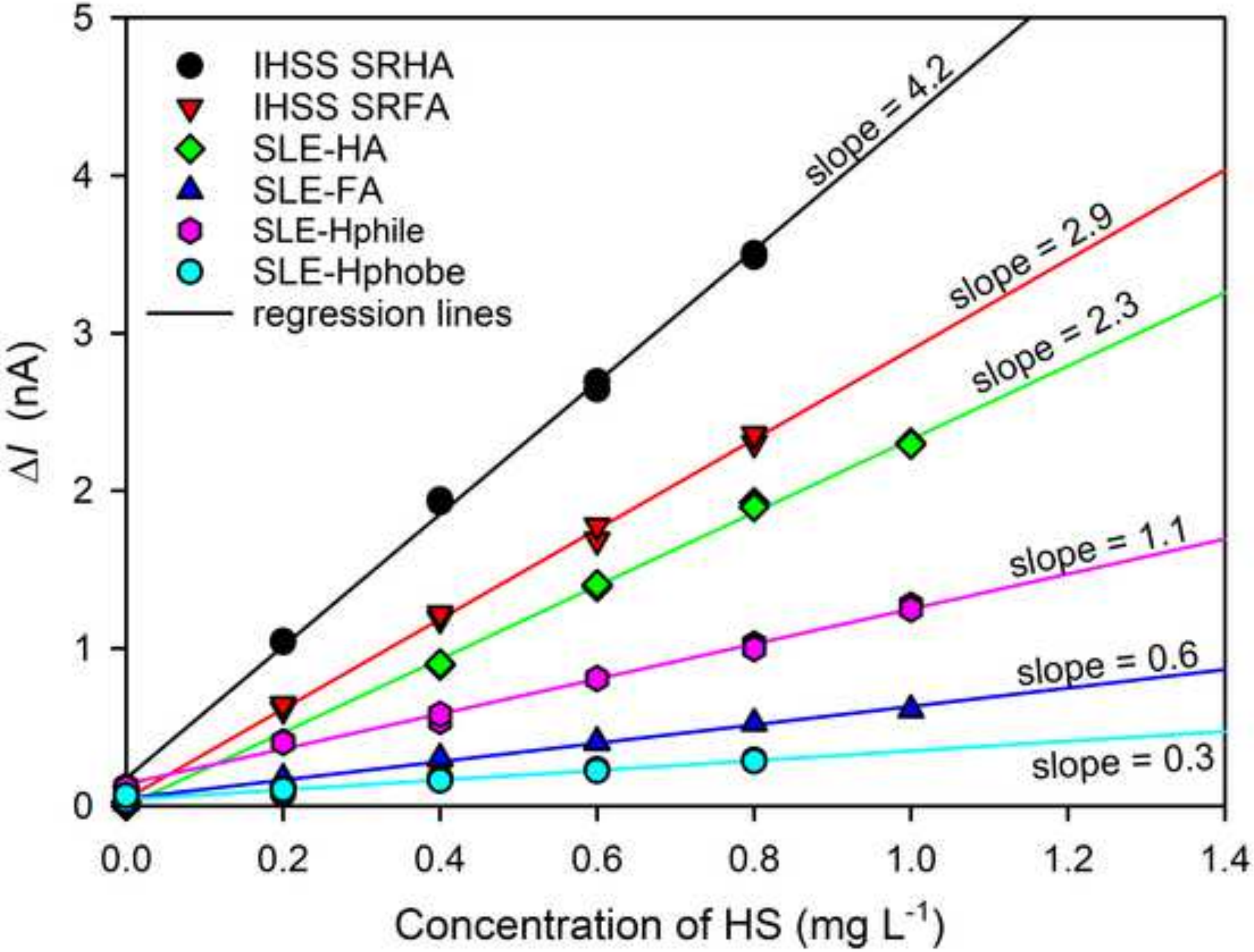
Figure 2. Calibration plots for different types of model HS (indicated in the internal legend) obtained with 60 s deposition time at 0.0 V using the new PB-HS method. ΔI is an average of the current from -0.75 to -0.80 V relative to a baseline scan at 2 s deposition time. The electrochemical parameters are given in **Table 1**.

Figure 3. Distribution of HA_{eq} (A) and its DOC-normalized HA_{eq} (B) in the vertical salinity gradient, obtained using the new PB-HS method for samples collected over different sampling campaigns (indicated in the graph by year and month).

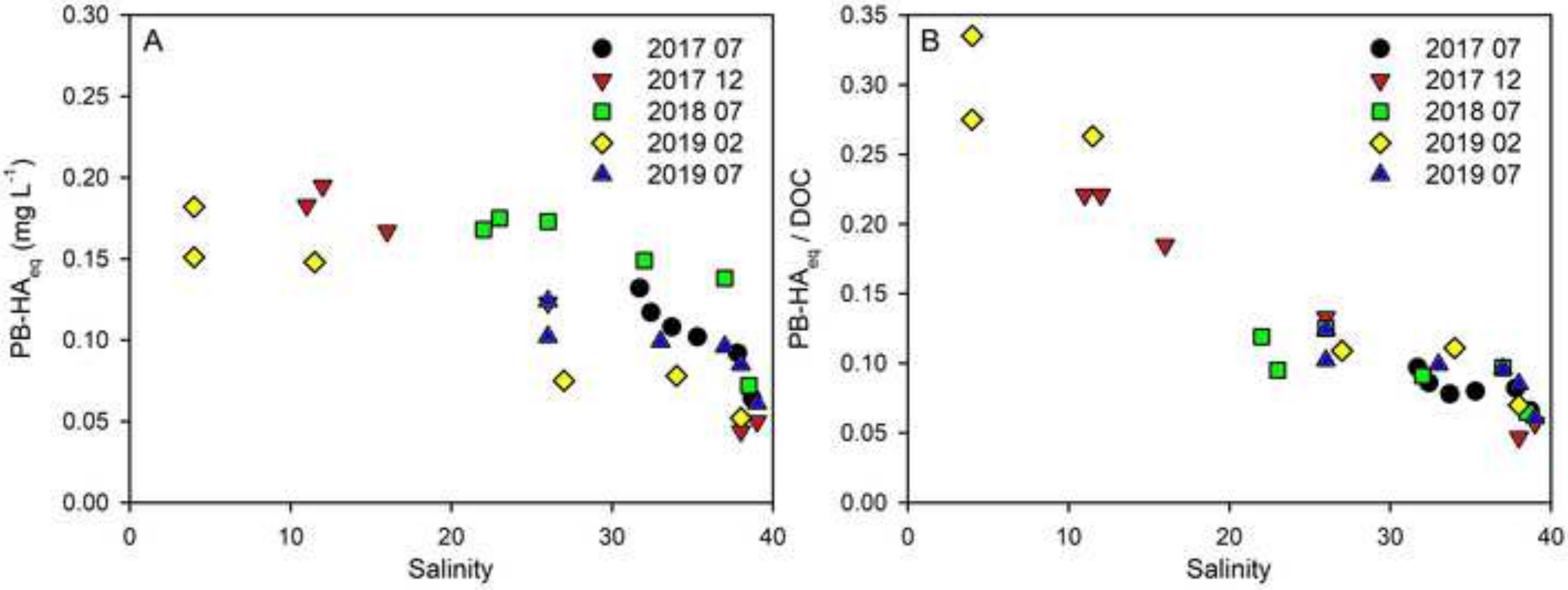
Figure 4. Relationship between HA_{eq} concentration obtained using the PB-HS method and the absorption coefficient measured at 254 nm for samples collected in the vertical salinity gradient over different sampling campaigns (indicated in the graph by year and month).

Figure 5. Relationship between HA_{eq} concentrations obtained using the PB-HS and Mo-HS methods for samples collected in the vertical salinity gradient over different sampling campaigns (indicated in the graph by year and month).


t_{dep} = 60 s
t_{dep} = 2 s
SRHA (mg L^{-1}):
0
0.2
0.4
0.6
0.8
1.0
-10
-8
-6
-4
-2
0
I (nA)
0.0
-0.2
-0.4
-0.6
-0.8
E (V)



ΔI (nA)
Concentration of HS (mg L-1)
0
1
2
3
4
5
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
● IHSS SRHA
▼ IHSS SRFA
◆ SLE-HA
▲ SLE-FA
⬢ SLE-Hphile
● SLE-Hphobe
— regression lines
slope = 4.2
slope = 2.9
slope = 2.3
slope = 1.1
slope = 0.6
slope = 0.3


A
B
2017 07
2017 12
2018 07
2019 02
2019 07
PB-HA$_{eq}$ (mg L^{-1})
PB-HA$_{eq}$ / DOC
Salinity
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0
10
20
30
40

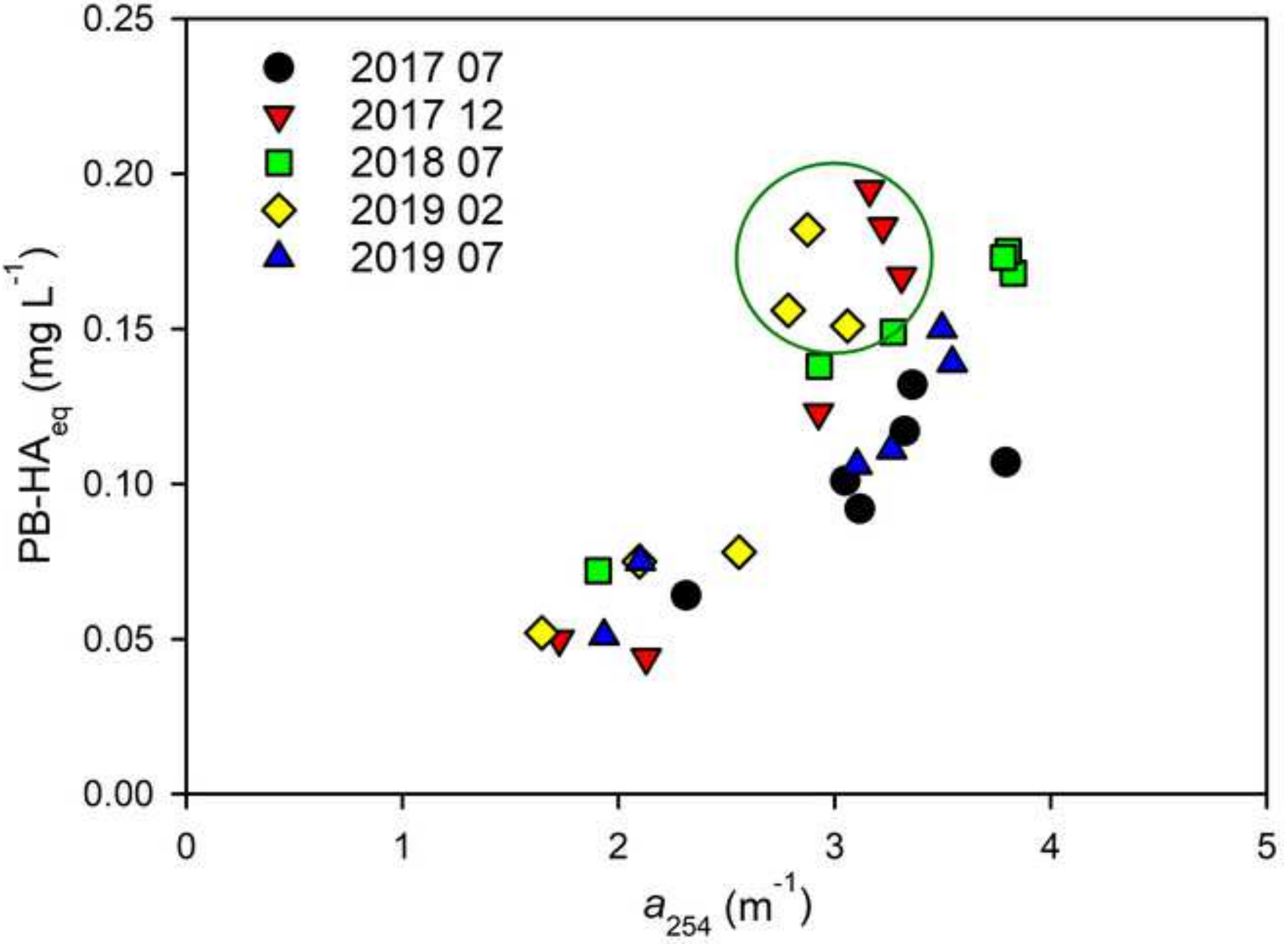
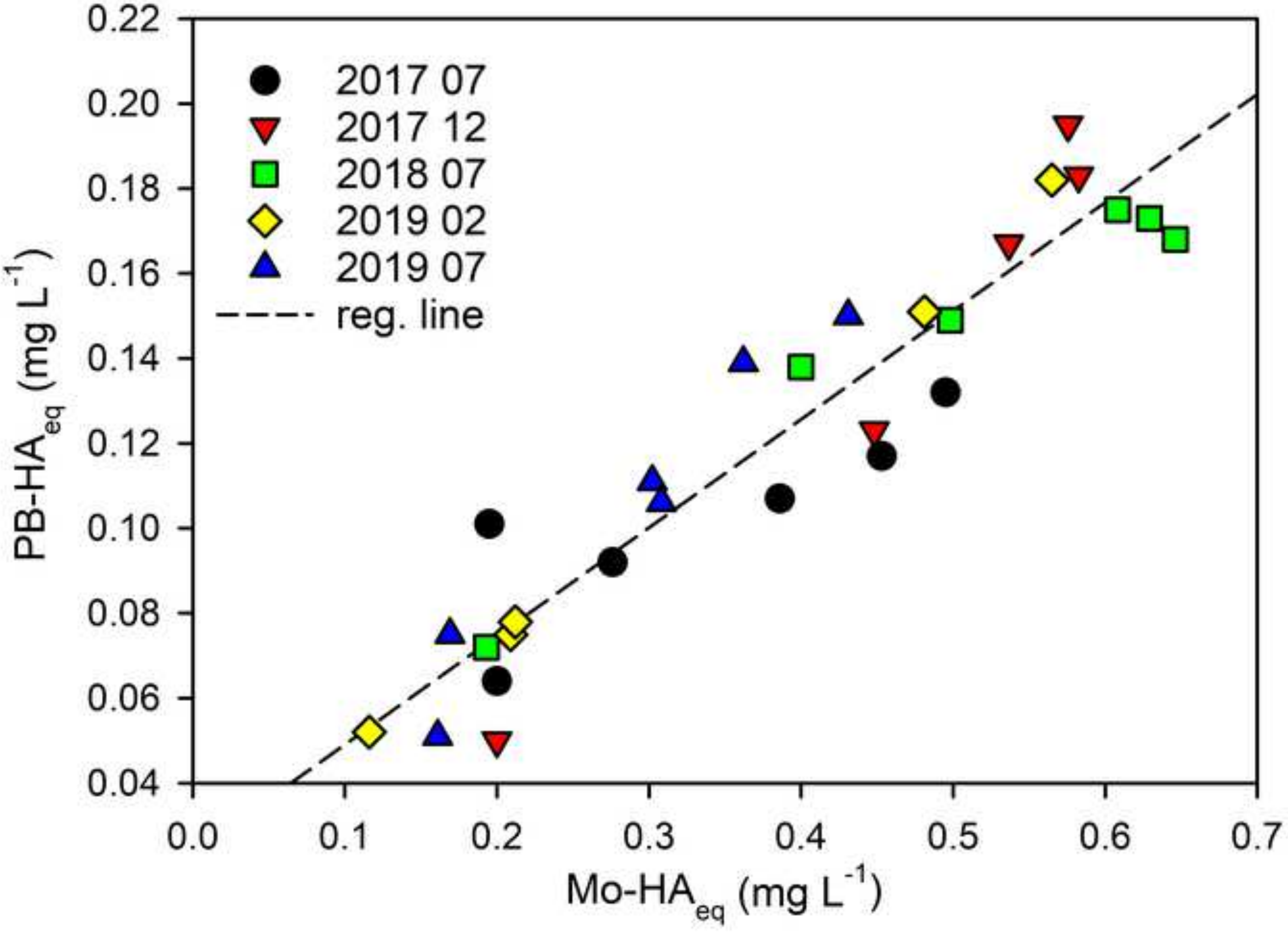

0.25
0.20
0.15
0.10
0.05
0.00
PB-HA$_{eq}$ (mg L^{-1})
0
1
2
3
4
5
a_{254} (m^{-1})
● 2017 07
▼ 2017 12
■ 2018 07
◆ 2019 02
▲ 2019 07

Figure 5


0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
PB-HA$_{eq}$ (mg L^{-1})
2017 07
2017 12
2018 07
2019 02
2019 07
reg. line
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Mo-HA$_{eq}$ (mg L^{-1})

Supplementary Material