Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

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27 December 2025

Assessment of bioaccumulation of glyphosate and aminomethylphosphonic acid in marine mussels using capillary electrophoresis with light-emitting diode-induced fluorescence detection

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ABSTRACT

Glyphosate or *N*-(phosphonomethyl)glycine, widely used as herbicide in agriculture to control weeds and to facilitate harvesting, has been included in Group 2A pollutants (probably carcinogenic to humans) by the International Agency for Research on Cancer (IARC). In intensive agricultural areas, runoff and soil leaching are likely to drive glyphosate to surface waters, where the compound is often detected together with its main microbial metabolite, aminomethylphosphonic acid (AMPA).

In the present study a method based on capillary electrophoresis coupled with light-emitting diode-induced fluorescence detection has been developed and validated for the determination of the two compounds in whole soft mass of marine mussels (*Mytilus galloprovincialis*). The method is based on the acidic hydrolysis of lyophilized tissue using 6 M HCl (oven at 110 °C for 22 h) to release the target analytes; their subsequent derivatization using 4-fluoro-7-nitro-2,1,3-benzoxadiazole, was found to be suitable for the sensitive fluorescence detection. To achieve optimum separation of the analytes from the matrix and degradation reagent interferences, the background electrolyte constituted by borate buffer (pH 9.2, 30 mM) was supplemented with 10 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin. The method was validated for linearity, precision, accuracy, robustness and sensitivity showing LOQ of 0.2 and 1.0 µg/g in fresh tissues, for AMPA and glyphosate, respectively; the recovery values ranged within 88.5 – 94.6% for glyphosate and 70.4 – 76.6% for AMPA. Experimental samples of Mediterranean mussels *M. galloprovincialis* treated with 100 µg/L or 500 µg/L of both glyphosate and AMPA, showed a dose dependent bioaccumulation of the compounds reaching maximum level of 77.0 µg/g and 11.3 µg/g of AMPA and glyphosate, respectively. The study demonstrates for the first time *M. galloprovincialis* as potential sentinel organisms for the environmental occurrence of these small amphoteric pollutants.

1. Introduction

Mussels are ideal bioindicators of the environmental quality of coastal marine ecosystems given their peculiar biological and ecological features such as: (i) sessility, making them permanently located to a limited and defined area; (ii) size, suitable for easy collection and storage together with enough availability of material per individual, useful for the chemical and bi-

ological analysis; (iii) sensitivity to several pollutant chemicals combined with the resistance to the same compounds, enabling their viability in culture for ecotoxicological experiments; (iv) feeding way, based on filtering large volume of seawater allowing for accumulation of the pollutant chemicals [1]. Several field-based and laboratory studies involve mussels as sentinel organisms in monitoring environmental pollution by different contaminants such as polychlorinated biphenyls and polyaromatic hydrocarbons, whose uptake, given their high hydrophobicity, is ascribed to the passive diffusion/partition from the dissolved phase to the external surfaces of the organism. In the bioaccumulation of other pollutants such as trace metals and ionizable chemicals (*e.g.,*

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pharmaceuticals), more complex mechanisms are thought to be involved [1,2].

Glyphosate or *N*-(phosphonomethyl)glycine is a small ampho-teric molecule widely used as herbicide in agriculture to control weeds and to facilitate harvesting. In intensive agricultural areas, runoff and soil leaching are likely to drive glyphosate to surface waters, where the compound is often detected together with its main microbial metabolite, aminomethylphosphonic acid (AMPA) [3]. Glyphosate has been included in Group 2A pollutants (probably carcinogenic to humans) by the International Agency for Research on Cancer (IARC) [4], thus an intensive research activity is nowa-days focused on its assessment in food and environmental samples.

The European Food Safety Authority (EFSA) has set the maxi-mum residue levels (MRLs) of glyphosate and AMPA in several an-imal commodities (*e.g.,* muscle, fat tissue, liver of swine, bovine, and sheep), ranging within 0.1 – 10 µg/g. Mussels are not included in the survey and no reports are available on the bioaccumulation and on their chemical determination either in laboratory or real samples [5]. Recent studies have highlighted a wide range of ad-verse effects of glyphosate and AMPA in aquatic species as inhibi-tion of acetylcholinesterase activity, increased oxidative stress, dis-ruption of pro-apoptotic signaling, stress responses, as well as em-bryotoxicity in aquatic invertebrates. In marine bivalves, environ-mentally realistic concentrations of the compounds affected sev-eral haemocyte parameters in exposed animals [3,6–9]. Notwith-standing the interest in evaluating the adverse biological effects of glyphosate and AMPA on aquatic organisms, their chemical deter-mination in mussels, either in laboratory or field samples, have not been reported.

The ionic character of glyphosate and AMPA, their low masses, high water solubility, low volatility, and lack of a significant chro-mophore, make the determination very challenging, especially in complex matrices. Liquid chromatography coupled to mass spec-trometry (LC-MS) is the most suited technique for analysis of glyphosate and related compounds in food, environmental and bio-logical samples [10]. To improve analytes retention in conventional reversed-phase chromatographic columns, a derivatization step is introduced *e.g.,* by using 9-fluorenylmethyloxycarbonyl chlorofor-mate (FMOC–Cl) [11], often in combination with solid phase ex-traction (SPE) as a clean-up procedure to remove matrix interfer-ences [12]. HILIC stationary phase with improved retention of polar analytes was applied in combination with MS/MS detection, to the analysis of glyphosate avoiding derivatization, in animal origin ma-trices upon SPE clean-up steps [13]. Direct analysis of glyphosate in matrices of animal origin, was proposed by Chiesa et al., upon sim-ple solid-liquid extraction using a mixture of methanol/formic acid, followed by ion chromatography hyphenated with high resolution mass spectrometry (Orbitrap) [14].

Capillary electrophoresis (CE) is a useful approach in the anal-ysis of glyphosate and metabolites; in capillary zone electrophore-sis (CZE) mode, the ionic solutes in a suitable background elec-trolyte (BGE) upon the application of an electric field, undergo to differential migration according to their charge-to-mass ratio. By performing the experiments in a fused-silica capillary with a small inner diameter (typically 50 or 75 µm), the band broadening of the analytes is very limited allowing high separation efficiency and orthogonal separation mechanism with respect to chromato-graphic methods [15]. The intrinsic poor sensitivity of CE due to the small injection volume, and to the short path (the capillary di-ameter itself) of the conventional on-column optical detection, is the most serious limitation in CE trace analysis. To face this issue, CE coupled with electrochemiluminescence detection was success-fully applied to the analysis of glyphosate and AMPA in soybeans, water and fruit samples achieving LOD of 0.6 µg/g [16,17]. The high sensitivity of laser-induced fluorescence (LIF) detection has allowed determination of glyphosate- and AMPA-fluorescein isothiocyanate

(FITC) derivatives in environmental [18,19] and food samples (veg-etables) in microfluidic format [20], achieving LOD in the order of tenth of µg/L. CE-LIF of glyphosate and AMPA in water samples was also approached by 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as the derivatization reagent, which provided the opportunity for the in-capillary derivatization due to the favorable reaction kinetic. Under optimized conditions, sensitivity of about 2 and 20 µg/L for AMPA and glyphosate respectively, was achieved [21].

The present study provides an original CZE-based methodology involving a simplified sample preparation based on acidic hydrol-ysis of lyophilized tissue of marine mussels (*Mytilus galloprovin-cialis*) to assess accumulation of glyphosate and AMPA, by *in-vivo* experiments. To achieve adequate sensitivity and avoid sample pre-concentration steps, light-emitting diode-induced fluorescence (LEDIF) detection, was applied upon derivatization with NBD-F of the marine mussels whole soft mass extracts. The obtained results represent the first evidence on the bioaccumulation of glyphosate and AMPA suggesting the potential of the Mediterranean mussels as sentinel organisms for the environmental occurrence of these pollutants.

2. Materials and methods

2.1. Standards and chemicals

The reference compound glyphosate and the cyclodextrins used as additives for CE separation, *i.e.,* β-cyclodextrin (βCD), (2-hydroxypropyl)-β-cyclodextrin (HP-βCD), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DM-βCD), heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-βCD), the surfactant sodium dodecyl sulfate (SDS) and the derivatization reagent fluorescein isothiocyanate (FITC), were from Sigma-Aldrich (Milano, Italy). Aminomethylphos-phonic acid (AMPA) was from Acros Organics (Thermo Fisher, Karlsruhe, Germany) and the derivatization reagent 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was from TCI Europe (Zwijn-drecht, Belgium). The components of the electrophoretic buffers and those used for preparation of the solutions for acidic hy-drolysis of mussel tissue samples, *i.e.,* sodium tetraborate, boric acid, hydrochloric acid (37%), sodium hydroxide, acetonitrile (HPLC grade), were purchased from Sigma-Aldrich. Ultrapure water for the preparation of the running buffers, samples and standard solutions was obtained by the Elix System (Millipore, Billerica, MA, USA).

Stock solutions of either glyphosate and AMPA for spiking and bioaccumulation experiments were prepared in ultrapure water, aliquoted and stored at −20 °C in the dark for one week. To achieve the solutions at different concentration of the pollutants for the mussels treatment, suitable aliquots of the stock solutions were diluted in sterile artificial seawater and spiked into the tissue preparations or spilled in vessels.

2.2. Experimental animals and holding conditions

Mediterranean mussels (*Mytilus galloprovincialis*) of commercial size (4–6 cm in length) were obtained from a government certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy). They were transferred to the laboratory in seawater tanks and acclimated in aquaria containing 35-psu filtered seawater at 16 °C with contin-uous aeration (>90% oxygen saturation), to support a reasonably good health status. During acclimatization, mussels were fed once a day with a commercial algal slurry (Koral, Xaqua). Before start-ing the experiments, mussels were immediately analyzed to assess their good initial health status employing the lysosomal membrane stability (neutral red retention assay) [22] (data not shown).
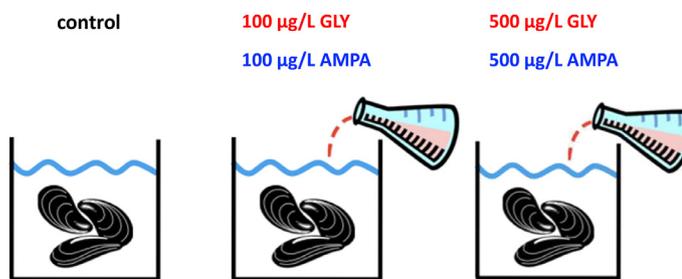
Fig. 1. Scheme of the bioaccumulation experiments with *in-vivo* exposed mussels. Mussels (20 animals each group) were treated with glyphosate and AMPA under following conditions: 3-days acclimatization; 7-days exposure to pollutants; 35-psu seawater; continuous aeration; constant water temperature (16 °C).

2.2.1. Spiking experiments with mussel total soft tissues

Whole mussel soft mass was dissected from 5 groups of unexposed animals (5 animal/group). Pooled samples (20.43 g fresh weight) were spiked with 1 mL of solution containing either glyphosate and AMPA at 25 µg/mL concentrations and homogenized using a UltraTurrax system (IKA, Staufen, Germany). Homogenates were frozen at −20 °C and lyophilized (2.90 g dry weight). A negative control was obtained by spiking additional sample pools with 1 mL of sterile artificial seawater.

2.2.2. Bioaccumulation experiments with in-vivo *exposed mussels*

Mussels were randomly selected and divided in groups of 20 animals each and transferred to vessels containing 20 L of water. One liter of seawater per mussel is the suitable volume to avoid overloading and prevent the onset of unfavorable health conditions. Mussels were treated for 7 days with 100 µg/L or 500 µg/L of both glyphosate and AMPA (Fig. 1). A group of unexposed (control) mussels was maintained in parallel to the treatment groups. Seawater was renewed each day and the chemicals added from stock solutions (prepared as described above) along with mussel feeding.

At the end of the treatment period, whole soft mass was dissected and washed in 35-psu sterile artificial seawater to be processed (preparation of homogenates and lyophilization) for chemical analyses as described below. There was no mortality during the exposure period. We established independent replicates within each treatment group by considering the 3 vessels as the operative replication level ($n = 3$). Bioaccumulation of glyphosate and AMPA was assessed on pooled samples, each pool consisting of whole soft mass from 5 animals.

2.3. CE instrumentation and analysis

The instrument for CE analysis was a G1600 HP³DCE from Agilent Technologies (Waldbronn, Germany) using the software Rev. A. 09. 01. Agilent Chemstation. Fused-silica capillaries (CM Scientific, Dublin, Ireland) were 48.5 cm total length and 21.5 cm to the detector, with the inner diameter (i.d.) of 50 µm (outer diameter 363 µm). New capillaries were conditioned by flushing in the following order, sodium hydroxide 1 M, sodium hydroxide 0.1 M and water (10 min each). The analysis was performed by using a BGE composed of borate buffer 30 mM (pH 9.2), supplemented with 10 mM DM-βCD. Between the injections the washing/conditioning cycle of the capillary was as follows: 3 min water and 3 min BGE. The injection of the sample was performed by hydrodynamic mode at 50 mbar for 20 s. The applied voltage was constant at 20 kV and the cartridge temperature was set at 25 °C. Detection was done using the ZetaLIF™ LED detector (LED-Induced Fluorescence Detector, LEDIF) by Adelis SAS (Labège, France) with 480 nm excitation wavelength and collecting the fluorescence emission in the range 515 – 760 nm.

2.4. Sample preparation

Aliquots of about 40 - 45 mg of the lyophilized mussel samples were treated with 1.0 mL of 6 M HCl in 4 mL vials (Clear Vial, Supelco, Milan, Italy) with PTFE/silicone septa. The mixture was ultrasonicated for 5 min and before capping, a nitrogen stream was gently fluxed. Hydrolysis was carried out in oven at 110 °C for 22 h, afterward, the mixture was neutralized using 6 M KOH; the solution was quantitatively transferred to a volumetric flask to adjust the volume at 5.0 mL with water. The sample was filtered with a 0.45 µm syringe filter and stored at −80 °C.

2.5. Derivatization

In a 1.5 mL plastic conic vial, an aliquot of 10 µL of the hydrolyzed sample was transferred and mixed with a 50 µL aliquot of borate buffer 10 mM, pH 9.2 and 25 µL of NBD-F solution (5 mM in acetonitrile); the mixture was stirred for 30 s and kept at 80 °C in a heating module (Reacti-Therm, by Pierce, Rockford, IL, USA) for 5 min, then after cooling in an ice bath, a 85 µL volume of water was added. The obtained final sample was stirred and injected into the CE apparatus.

2.6. Validation

Linearity was established in aqueous standard solutions in the range 0.070 – 2.00 µg/mL and 0.0100 – 0.500 µg/mL, for glyphosate and AMPA, respectively. Sensitivity was estimated by progressive dilution of standard solutions of both the analytes; the LOD value was established as the signal-to-noise ratio (S/N) = 3, and the LOQ was S/N = 10, where the noise was assumed as the distribution of the response at zero analyte concentration.

The precision, as degree of repeatability of migration time and peak area of the analytes in a representative sample was evaluated as RSD%. The recovery was estimated by quantifying the two compounds in three independent samples ($n = 3$) obtained by spiking with glyphosate (40, 20 and 10 µg/g) and AMPA (14, 7 and 3.5 µg/g) the lyophilized tissue of a control sample (mussels unexposed to the pollutants), before the hydrolysis procedure; from the same samples the recovery repeatability was also calculated. The robustness study was carried out by quantifying the two analytes in a representative sample using CE conditions involving parameters which were separately changed over an established range around their optimized values. In detail, the concentration of the BGE constituted of borate buffer was varied within 30 ± 2 mM, the concentration of DM-βCD was varied within 10 ± 1 mM, and the temperature of the cartridge was varied within 25 ± 1 °C.

2.7. Analysis of the samples

Quantitation of glyphosate and AMPA was carried out to assess the occurrence of bioaccumulation in experimental samples prepared as described (see Section 2.2.2. and Fig. 1) and subjected to the developed and validated method. Quantitative determinations were done in triplicate by interpolating the peak area response *versus* the calibration graphs established within the same day. Analysis of control samples was also conducted for comparison.

3. Results and discussion

Because of the lack of a significant chromophore, the CE determination of glyphosate and AMPA by optical detection requires derivatization. In addition, the application of pre-concentration approaches (either off-line or on-line) is often necessary to obtain the suitable sensitivity in investigations on residues determination in
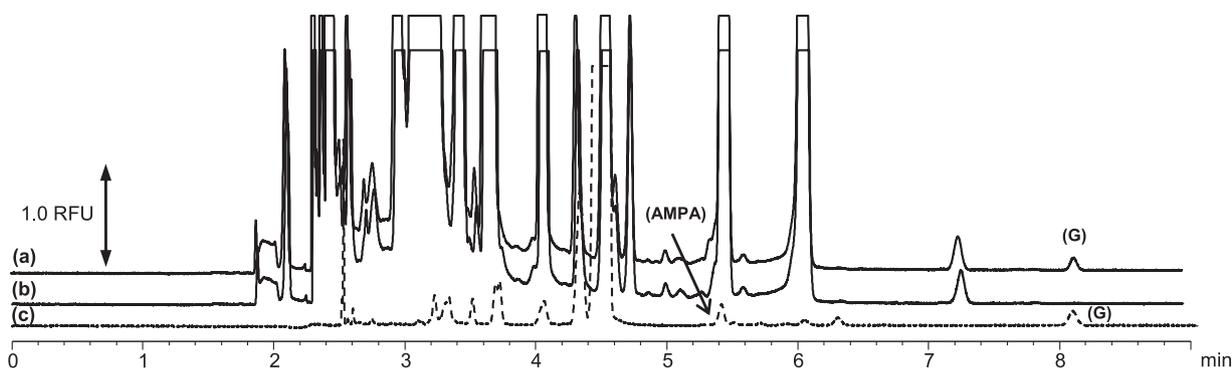
Fig. 2. (a) Electropherogram of a control sample (lyophilized mussels cultured in the absence of glyphosate and AMPA) subjected to acidic hydrolysis, spiked with the two analytes (0.4 and 0.08 μg/mL, respectively) and derivatized with NBD-F; (**b**) control sample; (**c**) standard solution of glyphosate and AMPA at the same concentration. CE conditions. BGE: borate buffer (30 mM, pH 9.2). Fused-silica capillary (50 μm, i.d.), 21.5 cm length to the detector. Voltage 20 kV; temperature 25 °C; hydrodynamic injection at 50 mbar x 20 s; LEDIF detection (λexc 480 nm). Symbols: AMPA is the NBD-F derivative of AMPA; G is the NBD-F derivative of glyphosate.

environmental and food samples. In a previous our study, a field-amplified sample injection and sweeping micellar electrokinetic chromatography (MEKC) using UV detection was applied to the determination of FMOC-–Cl derivatives of glyphosate and AMPA in wheat samples. A double SPE (C18/SAX, sorbents) procedure was necessary for the preliminary sample clean-up [23] as the sampling in the on-line pre-concentration was found to be affected by matrix composition [24].

To establish whether glyphosate and AMPA can be bioaccumulated in mussels, in the present study a CE method was designed for their quantification in the soft mass tissue minimizing the sample handling prior to the analysis. On-line pre-concentration approach was avoided and to achieve the necessary sensitivity, the LEDIF was selected as a suitable detection system. LEDs are alternative to lasers in fluorescence induced detection, with some advantages as they are less expensive, consume less energy and are more stable, maintaining similar high sensitivity [25].

3.1. Selection of the derivatization reagent and optimization of the separation

The most common derivatization reagents for the analysis of primary (AMPA) and secondary (glyphosate) amines, having the suitable fluorophore for the LEDIF detector (λ_{exc} = 480 nm) used in the present investigation, are FITC [18– 20] and NBD-F [21]. By preliminary experiments a higher fluorescence response of the FITC derivatives in comparison with NBD-F, was observed; however, the latter reagent produced less interferences by degradation products, and in addition the derivatization reaction was faster. The structures proposed for the NBD-F derivatives of glyphosate and AMPA (**Fig. S1**) are characterized by the presence of a phosphate group, thus under alkaline BGE, AMPA and glyphosate derivatives potentially possess a double and triple negative charge, respectively. In the presence of the high EOF obtained using alkaline BGE, the apparent mobility of the derivatives is expected to be lower than most of the matrix components and reagent degradation products; this behavior represented the rationale for the initially selected separation conditions.

CZE of NBD-F derivatized extracts was thus carried out in borate buffer (pH 9.2, 30 mM), one of the most suitable in analysis of anionic compounds. In Fig. 2**a** the electropherogram of a control sample is reported (lyophilized tissues of mussels cultured in the absence of the two analytes, subjected to the optimized extraction procedure based on acidic hydrolysis as described in experimental Section 2.4.), spiked before the NBD-F derivatization, with glyphosate and AMPA at 0.4 and 0.08 μg/mL, respectively. By comparison with the control sample (not spiked, Fig. 2**b**) and standard

solutions of glyphosate and AMPA (Fig. 2**c**), it can be observed that the CZE conditions did not allow the separation of AMPA from matrix interferences. Changes of the buffer concentration (borate 10 – 100 mM) and pH (8.0 – 10.0), did not provide any separation improvement. The addition of SDS (20 – 50 mM) to the BGE (30 mM borate buffer, pH 9.2) in MEKC mode led to completely different separation profiles, without resulting in selective resolution of the analytes from the matrix interferences.

Supplementing cyclodextrins (CDs) to CZE buffers is the most successful approach for chiral resolution and analysis of enantiomers and it is also applied for selectivity tuning in achiral separations [26,27]. Thus, with the aim at improving separation of the analytes from matrix interferences, neutral CDs were supplemented to the BGE. While the addition of βCD (5 mM) and HP-βCD (5 – 15 mM), did not improve separation, methylated CDs (DM-βCD and TM-βCD) showed to be more effective. However, with the use of TM-βCD (10 mM) as the additive to the BGE, only partial separation of the peak of AMPA derivative from the disturbances caused by matrix components was achieved (**Fig. S2**), whereas the addition of DM-βCD resulted in the complete resolution. Fig. 3 shows the electropherograms of the samples (spiked post-extraction, and control) in CE conditions using 10 mM DM-βCD as the BGE additive; higher CD concentration (15 mM) led to separation loss. In conclusion, the optimum conditions were as follow: borate buffer 30 mM (pH 9.2) supplemented with DM-βCD 10 mM; the separation was performed in a fused-silica capillary (50 μm, i.d.) with 21.5 cm length (to the LEDIF detector) at 20 kV and 25 °C.

3.2. Derivatization conditions

Since NBD-F reacts with primary and secondary amines in alkaline media [21], many potential interfering compounds from mussels samples (*e.g.,* amino acids, peptides and biogenic amines) are involved in the derivatization step. In real samples, the complex matrix environment and the possible high consumption of the reagent could prevent the completion of the reaction of the target analytes. Thus, the optimization of the derivatization conditions was carried out comparing the response of glyphosate and AMPA in standard solutions with that obtained in experimental samples prepared by spiking the control matrix with the two analytes (post-extraction spiking at 0.4 and 0.08 μg/mL of glyphosate and AMPA, respectively). The derivatization medium was a 10 mM borate buffer, pH 9.2, and the NBD-F concentration was 5 mM since lower concentrations resulted in incomplete reaction of the analytes in spiked samples. The reaction was carried out at room temperature (21 °C ± 2), at 50 and 80 °C for 5 min. In Fig. 4, the
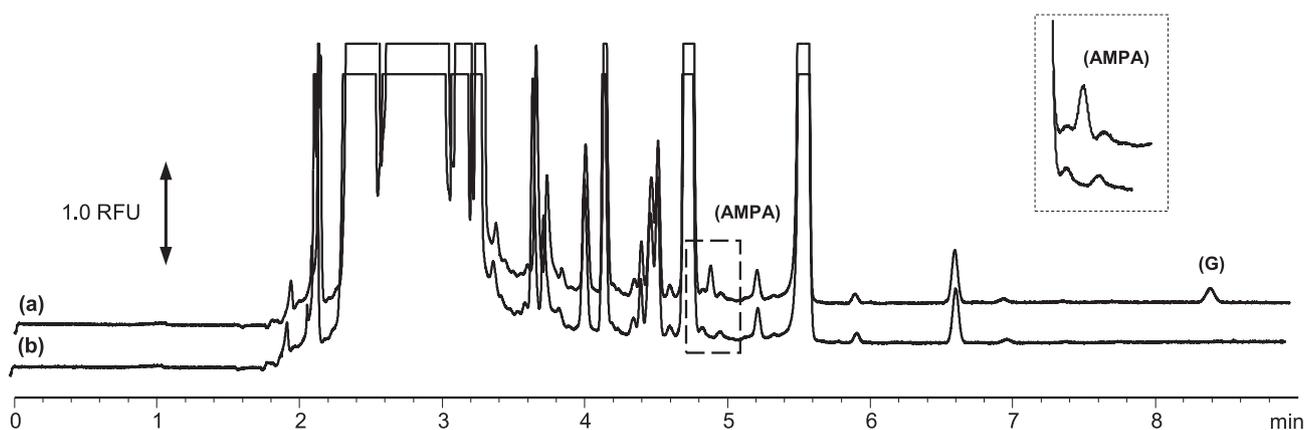
Fig. 3. (a) Electropherogram of a control sample spiked post extraction with glyphosate and AMPA as in Fig. 2a, and derivatized with NBD-F; (**b**) control sample. CE conditions as in Fig. 2, using borate buffer (30 mM, pH 9.2) solution supplemented with DM-βCD 10 mM. Other conditions and symbols as in Fig. 2. The inset reports the magnification of the peak of AMPA.
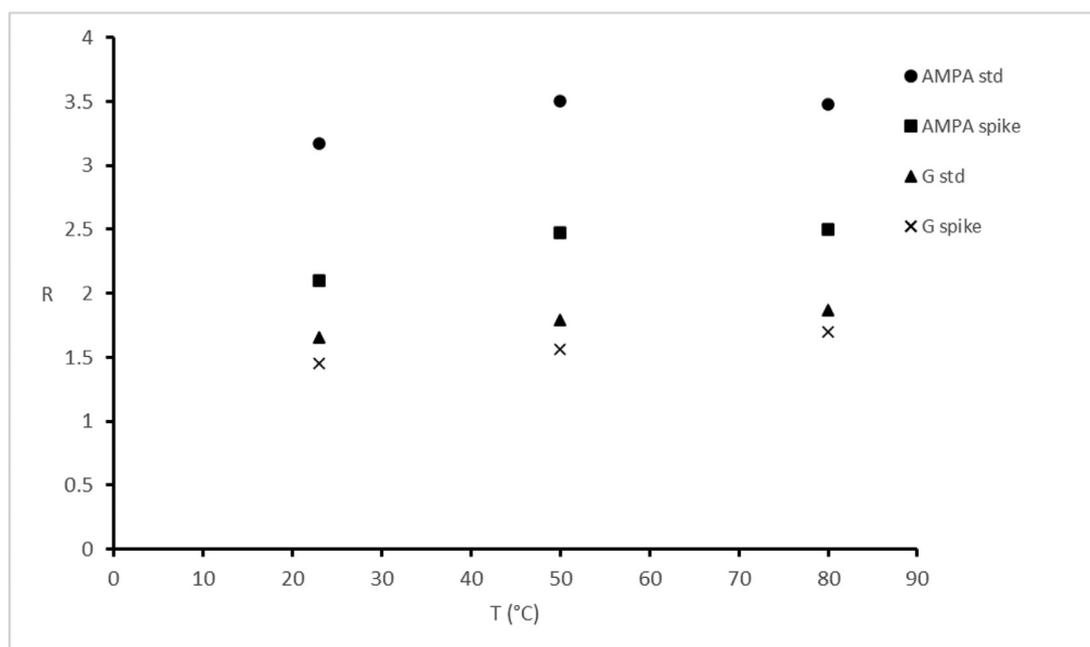

Fig. 4. Evolution of the fluorescence response (R, the peak area) of glyphosate (G) and AMPA derivatives in the reaction with NBD-F (5 mM) at different temperatures in standard aqueous solution (std) and in mussels matrix (spike). The data points are the mean of three measurements.

evolution of the obtained responses (peak area) is reported: as it can be seen, the reaction yield in spiked samples is maximized at the temperature of 80 °C with a different behavior for the two analytes. In particular, the response of glyphosate and AMPA in spiked samples approaches to about 90% and 70%, respectively compared to the standard solutions. The lower yield of AMPA can be reasonably ascribed to the migration of the derivative in a region of the electropherogram containing matrix interferences which partially hinder the fluorescence response. The effect of the pH of the reaction medium was evaluated in the range 7.5 – 9.2; no significant differences of the peak area of both the derivatives was observed, thus borate buffer at pH 9.2 was selected as the optimum since it is the native pH of the borax solution. It became apparent, during the optimization study, that the reaction reached completion in a very short time, and to establish a standardized protocol it was selected to stop the reaction by cooling the mixture in an ice bath after 5 min. Finally, the reaction mixture was diluted with water to avoid the sample overloading into the CE which led to a partial separation loss; the best compromise between complete separation and method sensitivity was obtained with a 1-to-1 dilution.

3.3. Sample preparation

The whole fresh soft mass removed from mussels exposed in laboratory experiments were subjected to a lyophilization procedure. Advantageously, the lyophilization is carried out on many samples that can be simultaneously dried in an unattended operation; in addition, the sample size is significantly reduced, and the lyophilized material does not need to be stored at low temperature. In order to evaluate if the two target analytes could be recovered from the lyophilized matrix, preliminary experiments were performed: fresh samples (wet weight 20.43 g) from mussels maintained in the absence of the two target compounds, were spiked with glyphosate and AMPA (25 μg each, see experimental Section 2.2.1.). Once lyophilization was completed, the sample weight was reduced to 2.90 g and the lyophilized material was used for optimization of extraction procedure. In many of the reports from the literature, to recover glyphosate and AMPA from matrix of animal origin, a solid-liquid extraction procedure using mixture of methanol/water acidified with formic acid is applied; then SPE is often employed to remove matrix interferences [12–

Table 1
Validation data.

Linearity and sensitivity	Glyphosate	AMPA
Range µg/mL	0.070 – 2.00	0.010 – 0.500
Equation	[a]Y = 2.16x - 0.0585	[a]Y = 15.9x + 0.0450
R^2	0.9998	0.9998
LOQ ([b]RSD%) µg/mL	0.07 (3.88)	0.01 (2.30)
LOD µg/mL	0.03	0.003
[c]LOQ$_{sample}$ µg/g	9.0	1.7
Recovery ([b]RSD%)		
[d]Low level	88.5% (7.21)	76.6% (8.34)
[e]Middle level	92.3% (5.42)	73.5% (5.50)
[f]High level	94.6% (5.63)	70.4% (5.25)

(a) Y is the LEDIF response (peak area); x is the concentration in µg/mL.
(b) $n = 3$.
(c) LOQ$_{sample}$ was estimated by considering the minimum amount quantifiable (Signal-to-Noise = 10) in lyophilized spiked sample (dry weight).
(d) glyphosate 10 µg/g; AMPA 3.5 µg/g.
(e) glyphosate 20 µg/g; AMPA 7 µg/g.
(f) glyphosate 40 µg/g; AMPA 14 µg/g.

14]. When similar conditions were applied to the lyophilized tissue, no recovery at the best of the method sensitivity was observed, likely because of the strong retention of the compounds by the dried matrix. The release of the analytes was achieved by subjecting the lyophilized tissue to acidic hydrolysis as it is applied in the compositional analysis of amino acids in mussels and other matrices (6 M HCl in oven at 110 °C for 22 h) [28]. Stability experiments were carried out on glyphosate and AMPA standard solutions subjected to the same conditions applied for the acidic hydrolysis. The complete recovery of both the compounds suggested that the proposed extraction approach did not introduce loss of the analytes for degradation. The combination of lyophilization and acidic hydrolysis of the samples can be considered as an effective procedure with the practical advantage to involve very limited handling operations while keeping unaltered the analytes content.

3.4. Method validation data

Validation data are reported in Table 1; the LODs values were in line with those reported using NBD-F as derivatization reagent and LIF detection [21]; FITC derivatives showed a slightly higher (5-fold) LIF response in analysis of both glyphosate and AMPA [19], however the procedure was not applied in quantitation of the analytes in complex samples. The present method is addressed to the determination of the analytes in mussel tissues, thus the sensitivity was also assessed by spiking experiments on control samples in order to establish the minimum amount quantifiable in real conditions. The LOQ values related to the weight of the lyophilized tissue (dry weight) were found to be 1.7 µg/g and 9.0 µg/g for AMPA and glyphosate, respectively (Table 1). As the weight loss observed in the samples due to the lyophilization process was found to be about 90%, the sensitivity values normalized to the real weight of the fresh tissue, resulted in LOQ of 0.2 µg/g for AMPA and 1.0 µg/g for glyphosate. These values are in the same order of those obtained using CE with electrochemiluminescence detection in determination of AMPA and glyphosate in vegetables [16]. Higher sensitivity has been reported using sophisticated techniques such as CE-TOF-MS in beer samples [29] and ion chromatography-high resolution mass spectrometry in animal tissues [14]. Interestingly, the latter study reports a survey on the LOQs obtained using separation methods hyphenated to mass spectrometry, showing in matri-

ces of animal origin, values ranging within 0.01 – 100 µg/g thus comprising the sensitivity of the present CE-LEDIF method [14]. Eventually, the sensitivity achieved with the proposed affordable approach, complies with the MRLs reported in EFSA survey for several animal commodities (0.1 – 10 µg/g) [5] and was found to be suitable for the intended purposes, that is to establish the occurrence of bioaccumulation of glyphosate and AMPA in mussels. Based on six replicate analyses of spiked sample (20 µg/g and 7 µg/g of glyphosate and AMPA, respectively), the RSD% values of the peak area were 6.2% (glyphosate) and 4.8% (AMPA). The very good values of RSD% of migration time (2.5% and 1.2% for glyphosate and AMPA, respectively), allowed for the unambiguous identification of the analytes by migration time comparison.

Recovery was evaluated for the two target analytes by spiking the lyophilized control matrix before acidic hydrolysis at three levels. As it can be seen in Table 1, the recovery of glyphosate and AMPA were higher than 88.5 and 70.4%, respectively. These values can be considered adequate taking into account the complexity of the analysed samples. The robustness of the method was assessed with respect to the content of the analytes in one of the spiked samples prepared for recovery evaluation (as in Table 1, middle level of spiking). The separation conditions applied in robustness were deliberately changed with respect to the optimum of some relevant parameters (borate buffer 30 ± 2 mM, DM-βCD 10 ± 1 mM, cartridge temperature 25 ± 1 °C). The found amount of both the analytes was within the confidence interval (α=0.05; $n = 3$) calculated around the content at the central level, demonstrating good method robustness.

3.5. Analysis of experimental samples

Glyphosate and AMPA can reach surface waters as the result of either runoff and soil leaching; their concentrations can vary along the seasons depending on rainfalls intensities from few µg/L to mg/L and have been detected in a number of different water bodies, including seawater [3]. In ecotoxicological studies performed on seawater culture media, glyphosate and AMPA were reported to range within about 10 – 1400 µg/L and 50 – 140 µg/L, respectively [30]. In previous studies on the risk assessment of both the pollutants to *M. galloprovincialis*, levels up to 1000 µg/L [6] and 100 µg/L [7–9] of glyphosate and AMPA, respectively, were considered as environmentally realistic in exposure experiments (7, 14, 21 days) [6–9]. Thus, in the present study, to establish the occurrence of bioaccumulation, the validated CE-LEDIF method was applied to experimental samples obtained in 7 days *in-vivo* exposures of *M. galloprovincialis* at two concentration levels of both the pollutants (100 µg/L and 500 µg/L), along with a group of unexposed (control) mussels (Fig. 1). The two compounds were not detected in the control samples, whereas they were found in all of the samples treated with 500 µg/L concentrations. However, while AMPA was quantified at a mean value of 33.16 µg/g (on dry weight), glyphosate with an exception, was only detected at trace level (Table 2). In the samples obtained when exposure involved 100 µg/L of the pollutants, only traces of AMPA were found.

Despite the relevant amount of experimental evidence on adverse effects of glyphosate and AMPA, [3, 6 - 9], to date no information on mussel accumulation and elimination potential for these compounds is given, as well as scarce data on wild aquatic species are reported. This represents a relevant bias in assessing the ecotoxicological impacts of glyphosate and its metabolites on marine species. While considering the difference in method sensitivity favorable to the determination of AMPA, the striking mismatch in detected tissue concentrations between the two compounds may derive from the possibility for glyphosate to be metabolized in mussels as it occurs in fish and mammals [31]. In mammals, glyphosate is metabolized by the animal CYP450 enzymes and by

Table 2

Amount* in µg/g (dry weight) of glyphosate and AMPA found in experimental mussels samples exposed for 7 days to 500 µg/L of both the pollutants.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
AMPA	20.3 ± 0.97	15.5 ± 0.78	44.2 ± 1.76	77.0 ± 3.70	18.5 ± 1.00	23.5 ± 1.28
Glyphosate	tr	tr	tr	11.3 ± 0.85	tr	tr

*Mean amount ($n = 3$).
tr, trace.

gut microorganisms bearing 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme activity within the shikimate pathway [31]. Lacking information on the mussel metabolic potential, a recent study on mussels experimentally exposed for 21 days to 10, 100, and 1000 µg/L glyphosate, has reported evidence of the activation of detoxification mechanisms and disruption of the molecular pathways controlling energy metabolism [8]. Part of these changes were further related to alterations occurring in digestive gland microbiota composition following glyphosate and AMPA exposures [9]. Therefore, the combination of physiological changes (either within the host or the microbiota component) and chemical features may contribute to a possible different tissue retention of glyphosate and AMPA. Future focused studies should address this issue and will also involve the analysis of extracts from real samples of the coastal marine ecosystems.

4. Conclusion

CE-LEDIF showed to be suitable for the analysis of glyphosate and AMPA in extracts of tissue of Mediterranean mussels, upon derivatization using NBD-F. Some relevant aspects that can be underlined as finding of the present investigation are the following: (i) lyophilization of animal tissue (mussels) and subsequent acidic hydrolysis represent a practical approach for storage and sample preparation; (ii) thanks to the wide opportunity for selectivity tuning, CE showed to be an affordable and green technique for analysis of complex samples; (iii) for the first time it has been shown that glyphosate and AMPA can bioaccumulate in marine mussels, thus *M. galloprovincialis* can be proposed as potential sentinel organisms for the environmental occurrence of these small amphoteric pollutants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests

CRediT authorship contribution statement

Roberto Gotti: Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition. **Jessica Fiori:** Investigation, Writing – original draft. **Sandra Furlanetto:** Software, Writing – review & editing. **Serena Orlandini:** Software, Writing – review & editing. **Marco Candela:** Conceptualization. **Silvia Franzellitti:** Conceptualization, Investigation, Writing – review & editing.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463452.

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