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CORSO DI LAUREA IN CHIMICA

NMR characterization of marine sediments in the Venice lagoon

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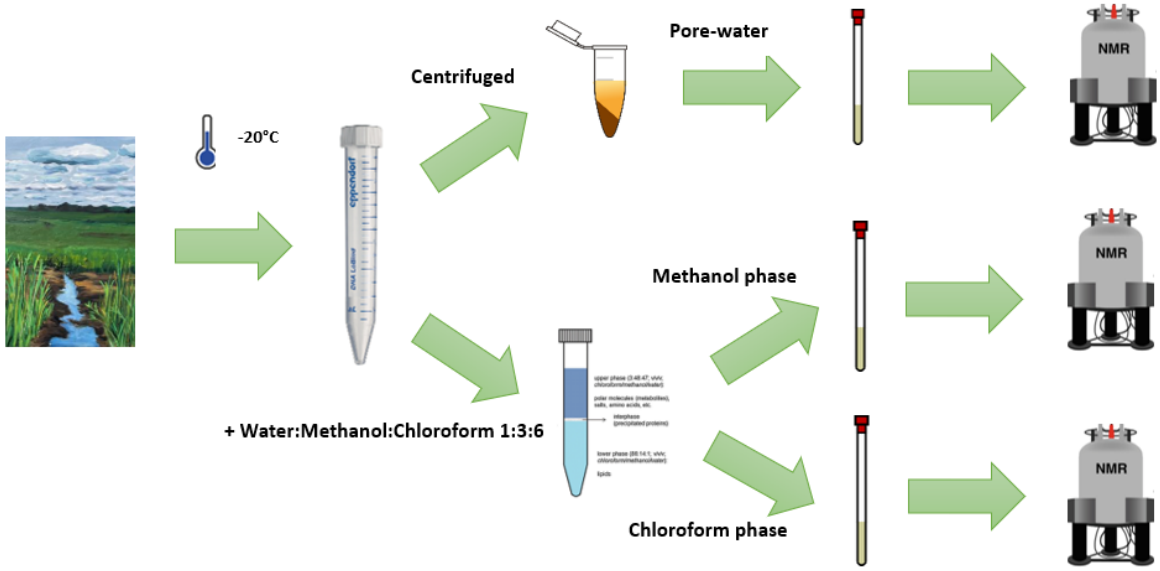
SUMMARY

ABSTRACT

This study investigates the metabolite composition of different salt marsh types in the Venice Lagoon. The aim is to identify metabolites that can indicate the metabolic presence and activity of microorganisms in those sediments.

A total of 10 samples were stored and processed to obtain a liquid phase of pore water and two other extraction phases in methanol and chloroform to detect hydrophilic and lipophilic molecules. Spectra were acquired for a subset of extracts with 1D ^{1}H NMR and 2D ^{1}H-^{1}H NMR techniques. After pre-processing, peaks were manually assigned using HMDB and GISSMO databases resulting in a total of 40 identifications. Despite minor technical setbacks, the methodologies presented here improved the quality of spectra compared to previous studies.

ABSTRACT GRAFICO



INTRODUCTION

Salt marshes environment

A marsh is a type of wetland, an area of land where water covers ground for extended periods of time. Unlike swamps, which are dominated by trees, marshlands are covered by grasses and other herbaceous plants. This structure, where plants grow densely above and below the ground with an extensive and continuous root system, traps nutrient efficiently and defends the soil against erosion or drying out. This helps plants growth and soil build up, accumulating CO_2 in the process. Indeed, wetlands store about five times more CO_2 than forests and as much as five hundred times more than oceans [1][2].

These ecosystems not only remove CO_2 from the atmosphere by storing carbon in their living and dead biomass through photosynthesis and microbial fixation, they also trap externally produced organic carbon suspended in tidal flows and terrestrial runoff, which they continually accrete within their sediments.

Because of their position where land and waters meet, marshlands are particularly exposed to climate change and human activities. Indeed, marshlands are disappearing at an alarming rate all over the world: three times faster than forests. Accordingly, wetland degradation releases enormous amounts of CO_2 and contributes to 5% of our global annual CO_2 emissions [2].

In this context, understanding marshland functioning becomes particularly important to prevent their disappearance and to inform their restoration.

Biological processes in marshlands

In marshlands, the carbon cycle starts mainly from two sources: atmospheric or water dissolved CO_2 and decomposition of plants [3].

Photoautotrophic organisms such as plants, phytoplankton and some types of bacteria fix CO_2, to create organic molecules (e.g., glucose), which are used to build up biomass in the form of large polymers (e.g., cellulose, lignin, proteins).

Dead biomass from primary producers is then processed by heterotrophic microorganisms that, break down large quantities of organic compounds to form important biomolecules, such as carbohydrates, lipids, amino acids and nucleotides. These molecules are fundamental to the survival and reproduction of heterotrophs and a valuable source of nutrients for primary

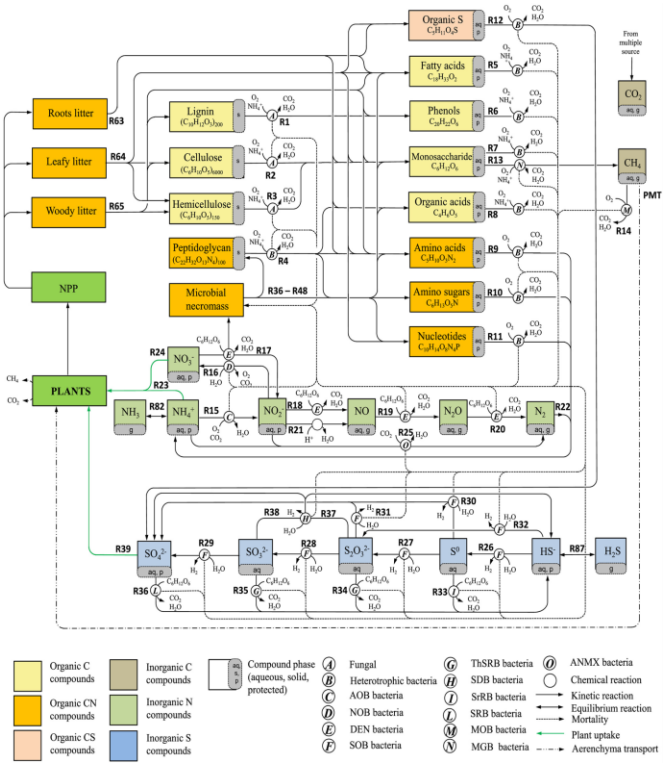


Figure 1: process and compound involved in carbon cycle [4].

producers. The main pathways are illustrated in Figure 1.
In this work, we focused on the metabolite fraction of salt marshes sediments, which is composed of a variety of carbohydrates and sugars, free and bound amino acids, fatty acids, lipids, phenolic compounds, aromatic compounds and carboxylic acids [5].
Metabolites are mostly products of microbial metabolism; information on metabolite composition and abundance can help understanding ongoing biological processes and the crucial role of microbial communities in marshland ecosystems [3].

Classes of compounds found in marine sediments.

Amino acids

Amino acids (AA) are defined as organic substances containing both an amino and a carboxylic acid group. Among the 300 AA in nature, only 20 (α-AA) serve as building blocks for proteins, while non-protein α-AA (e.g., ornithine, citrulline, homo-cysteine) and non-α AA (e.g., taurine and β-alanine) play important roles in cell metabolism and physiology (e.g., osmoregulation, hormone secretion, and gene expression) [6].

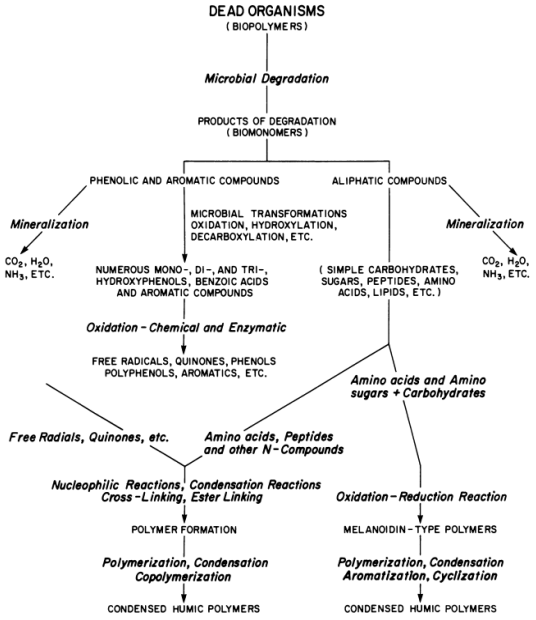


Figure 2: All classes and major specific metabolites produced by microorganisms.

In soil and sediments, most of the production of amino acids comes from organisms that can fix nitrogen compounds such as azotobacter, archaea and fungi or from the plants that host them. Nitrogen fixation starts with the conversion of N_2 into NH_3. This process is energetically expensive and consumes 16 moles of ATP per mole of N fixed [7]. Organisms can also oxidate AA to produce energy converting them to CO_2 and H_2O mainly via the Krebs cycle and the mitochondrial electron transport system [6].

Nucleotides

Nucleotides are another class of metabolites based on nitrogen, composed of a nitrogenous base, a pentose sugar, and a phosphate. They are building blocks for nucleic acid polymers, and free nucleotides play roles in cellular energy storage and provision, cellular signalling, as a source of phosphate groups used to modulate the activity of proteins and as enzymatic cofactors, often conducting redox reactions [8].
Nucleotides are synthesized *de novo* from phosphoribosyl-pyrophosphate (PRPP) a central molecule for thousands of metabolic pathways in all living organisms.

Carbohydrates

Carbohydrates are a classes of compound composed of carbon (C), hydrogen (H) and oxygen (O) atoms, usually with a hydrogen:oxygen atom ratio of 2:1 and with the empirical formula $C_x(H_2O)_x$

They are important constituents of all marine and terrestrial organisms, including microorganisms. They are a useful source of energy and serve as a base for cell structure. Most carbohydrates in the biosphere are polymers of varying complexities and include many groups of compounds such as cellulose, hemicellulose, starch, sugars, chitin, pectin, and agars.

Glucose is the carbohydrate most involved in all metabolic pathways because most of energy produced by microorganisms starts with it via glycolysis, producing in aerobic condition, 32 ATP molecules per each glucose molecule. It is also the building block of the two most abundant polysaccharides in nature: chitin and cellulose [9].

Chitin is a major component in the skeletal structure of lower animals, fungi, and many marine organisms, such as crustaceans and molluscs and is important for sugar and amino acid metabolism as well as for peptidoglycan biosynthesis [10].

Cellulose is synthesized mostly by plants. Half of plant cell wall is made of cellulose, so its principal function is structural. In soil and sediments, fungi, bacteria, and archaea mediate cellulose decomposition to glucose that is used as energy source for metabolism and reproduction. The end products of the enzymatic hydrolysis of cellulose are organic acids (e.g., acetic acid, succinic acid, formic acid, lactic acid, butyric acid), alcohols such as ethanol and alkanes such as methane. These low molecular weight organic acids and sugars further participate in a series of chemical and biochemical reactions [11].

Lipids

Lipids are a large and diverse group of naturally occurring organic compounds that includes not only fats and oils (such as esters of the trihydroxy alcohol glycerol and fatty acids), but also phospholipids, terpenoids and steroids [12].

Phospholipids are important components of biological membranes because of their amphipathic properties; their fatty acids can be saturated or unsaturated and their ratio determines membrane fluidity. The most common saturated compounds are palmitic acid and stearic acid; oleic acid and linoleic acid are the prevalent unsaturated fatty acids [13].

Among microorganisms, cell membrane structure can vary significantly: archaea have glycerol-1-phosphate phospholipids with ether-bond isoprenoid chains, while bacteria have glycerol-3-phosphate (G3P) esterified to fatty-acids. Although there are pathways for synthesis of isoprenoids and fatty acids in both domains [13][14].

Lipids are also important as energy source.

They are produced by all plants and animals; they are synthetized starting from acetyl-CoA and are degraded producing again acetyl-CoA.

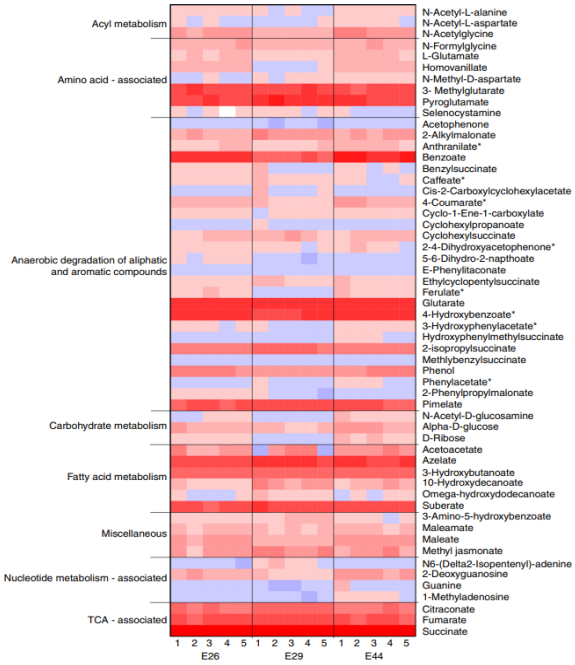
Among microorganisms, phytoplankton, photosynthesizing bacteria and protists are the most important producers of organic matter in the marine environment and contain significant amounts of lipids.

Lignin

Lignin is an important skeletal material in higher plants, so it is not synthesized by marine organisms; however, it has been identified in marine sediments. It is a three-dimensional high molecular weight polymer of various phenols.

Lignin is degraded by many organisms, thereby releasing several phenolic and polyphenolics compounds. Of the many degradation products of lignin, a few worth noting are vanillic acid, ferulic acid, vanillin, and hydroxy vanillin [15].

Figure 3: Specific metabolites associated with different bacteria for different metabolic pathway. [15]



Methods for the characterization of metabolites in marine sediments

Mass spectrometry

Mass spectrometry is a technique that uses molecular ions and their fragments to identify them. It is based on the ionization of sample molecules, followed by separation and detection of the resulting ions according to mass-to-charge ratio (m/z). Results are displayed in the form of a mass spectrum, which is a graphical representation of ion abundance versus m/z. Mass spectrometry provides information about the molecular mass of the sample molecule and the masses of its fragments, which can be used to determine the structure of molecules. Under given conditions, every molecule fragments in a unique manner, making molecular identification possible even in complex mixtures [16].

The most common ionization methods are:

- Electron impact ionization: a vaporized sample is passed through a beam of electrons and the high energy (70 eV) beam strips electrons from the sample molecules leaving a positively charged radical species. The molecular ion undergoes decomposition or rearrangement to produce fragment ions [17].
- Chemical ionization (CI): the sample is introduced to a chamber filled with excess reagent gas (such as methane). The reagent gas is ionized by electrons, forming a plasma with species such as CH_5^+, which react with the sample to form the pseudo molecular ion $[M+H]^+$. Because CI does not involve radical reactions, fragmentation of the sample occurs less [17].
- Electrospray ionization: allows for the sample to be non-volatile or thermally unstable. It is a soft ionization. In this technique, solvent droplets are created and shrunk via evaporation, until a coulombic explosion occurs, producing daughter droplets containing sample ions. One of the limitations is that the sample must be soluble [17].

Soft ionizations are a useful technique when considering biological molecules of large molecular mass, because this process does not fragment the macromolecules into smaller charged particles.

Mass spectrometers are often coupled to gas chromatography (GC) or liquid chromatography (LC) [16]. Both techniques separate molecules based upon their characteristic and their affinity to a stationary phase and are transported by a mobile phase. In GC, the mobile phase is a gas (usually inert and unreactive, such as helium or nitrogen) so the analysed molecules should be vaporized without decomposition and must be volatile below about 350 °C. In LC, the mobile phase is a liquid.
MS is used in biomedical research to provide rapid and sensitive qualitative and quantitative analysis of biomolecules and metabolites in cells, tissues, or organs [18]; it also has environmental applications.
Gas spectrometers can quantify volatile compounds at concentrations as low as 20 parts per trillion and these analysers can be used for water pollution monitoring.
Indeed, many published studies focus on water and sediment analysis.
For example, Berger et al. 2020 quantified contaminants in river sediment using gas chromatography-mass spectrometry instrumentation. The results indicated pollution by polycyclic aromatic hydrocarbons (PAHs), which are concomitant with petroleum contamination [19].
Mehinto et al. 2023 used non-targeted high resolution mass spectrometry to assess the quality of 70 marine sediment samples collected from five distinct coastal and offshore habitats of the Southern California Bight to expand the breadth of compounds monitored and evaluate the potential hazard of environmental mixtures [20].

NMR

Nuclear Magnetic Resonance (NMR) spectroscopy is used to determine the composition and structures of molecules in a sample. Nuclei can have multiple spins and are electrically charged, hence they possess magnetic moment and generate a magnetic field. In the presence of an external magnetic field, spins are not randomly distributed, and energy levels are defined.
When a second orthogonal magnetic field is applied with enough energy and the right frequency to promote a nucleus from a lower energy spin level to a higher one, energy transfer occurs from the instrument to the sample. When a molecule is analysed, all nuclei spin promotions are registered by the instrument and this frequency is a signature characteristic of the nucleus type and of its space interaction with other nuclei. This spectroscopy allows interaction between nuclei in a molecule to be detected and the molecular structure to be obtained from the spectra [21].
Compared to MS, NMR is a less expensive and a faster method of analysis. It has a high reproducibility and requires minimal sample preparation. On the other hand, NMR is less optimal for untargeted analysis and requires higher concentrations to detect metabolites [22].
It is a very useful approach to determine the chemical structure of organic molecules.
The analysis of complex mixtures by NMR has also been used for the characterisation of many biological matrices, such as extracts of microorganisms, mammalians, and plant tissues, but also clinical tissues and biofluids such as plasma, urine and cerebral

spinal fluid have been described, involving many fields from biomedicine to environmental sciences. NMR has been also applied to study the composition and structure of major biological elements in dissolved organic matter (DOM).

However, to date, studies that focus on the NMR characterization of sediments are scarce. The most relevant work comes from Christina A. Fox et al. 2018 and uses ^{1}H NMR spectroscopy and 2D ^{1}H-^{1}H TOCSY spectra to examine the effect of depth on the composition of pore-water DOM in anoxic sediments.

This study reports approximately the molecular class attributable to detected signals but no specific assignments are made, hinting at the challenges of sediment characterization. (Figure 4 and Table 1) [23].

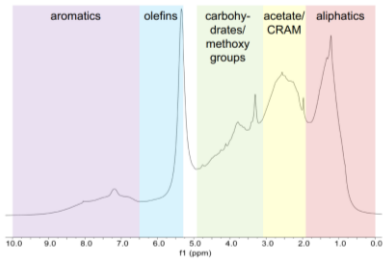


Figure 4: NMR spectra regions of the study [23].

Table 1: Defined regions and assignment [23].

δ_H (ppm)[a]	ID	Assignment	H/C ratio[b]	Compound Class	Group[c]
0.91–1.24	*C*	CH_3 in 6-deoxysugars[d]	1.67	carbohydrate	2
1.24–1.51	*E*	CH_2-C-CO(NHR)	1.58	protein & peptide	2
1.70–1.84	*H*	CH_3-C-SH	2.00	methanethiol	2
1.95–2.22	*J*	CH_3-C=O-NH[e]	1.67	*N*-acetyl amino sugar; acetate derivatives	1
2.72–2.80	*L*	CH_3-CH_2-C=O-NH[f]	1.58	amino sugar	2
2.94–3.15	*M*	CH_2-NH_2	1.58	protein & peptide	2
1.51–1.64	*G*		1.4	subsection of CRAM	1
2.22–2.72	*K*		1.4	subsection of CRAM	1
0.91–3.15	CRAM		1.4	CRAM, total	
3.15–4.14	*N*	HC-O	1.67	carbohydrate	2
4.91–7.00	*O*	HC=C	1.00	olefinic	
7.00–10.00	*P*	H-Ar	1.00	aromatic	

Common extraction methods

Extraction is an important step in the identification of metabolites. Several methods have been proposed to date, with different protocols and solvents.

Most studies, including those which analyse soil or sediments, extract metabolites depending on their differential affinity for polar and non-polar solvents.

Malak M. Tfaily et al. 2017 compare six methods of sequential organic matter extraction using three sample types, peat soil, spruce forest soil and river sediment. In total, four solvents were used in order of polarity: water (H_2O), methanol (MeOH), acetonitrile (ACN) and chloroform ($CHCl_3$) (Table 2) [24].

Table 2: Summary of the solvents used in each protocol [24].

	1st solvent	2nd solvent	3rd solvent	4th solvent
Protocol 1	H_2O	MeOH	$CHCl_3$	–
Protocol 2	H_2O	ACN	$CHCl_3$	–
Protocol 3	H_2O	MeOH:ACN (9:1)	$CHCl_3$	–
Protocol 4	H_2O	MeOH	ACN	$CHCl_3$
Protocol 5	H_2O	ACN	MeOH	$CHCl_3$
Protocol 6	H_2O	$CHCl_3$:MeOH (2:1)	–	–

Extraction protocol 6 provided the best results for river sediment extraction (Table 3), allowing more peaks to be obtained in the mass spectrum. Given these results, we assumed that this method would be the most efficient also for NMR analysis.

Table 3: number of peaks in total and in each solvent in MS [24].

H_2O	3089	2927	2941	3041	2898	2990
MeOH	1246			532	944	1837
ACN		2059	1737	1074	671	
$CHCl_3$	396	376	636	1304	1372	3832
Total	*4731*	*5362*	*5314*	*5951*	*58855*	*8659*

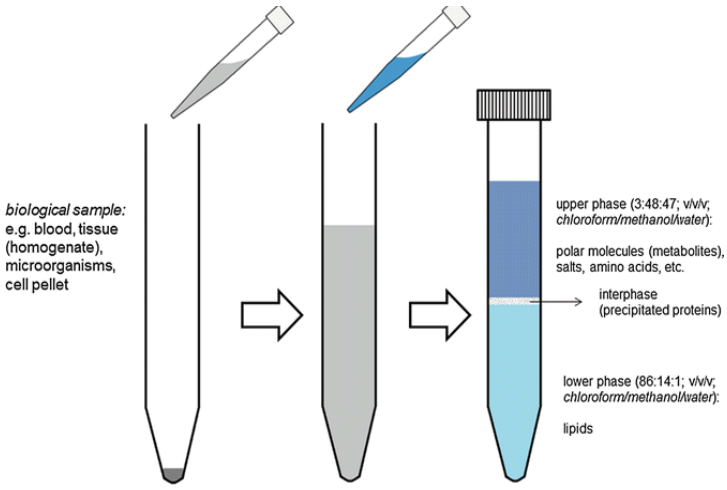


Figure 5: Graphical representation of extraction methods [25].

The use of two solvents with different polarity is common in metabolomics and it is widely used in extraction procedures such as the Folch method [25] and the Bligh and Dyer method [26].
The main differences between these two methods are the ratio of chloroform/methanol/water (2:1:0.75 in Folch and 1:1:0.9 in Bligh and Dyer method) and the volume of the solvent system (twenty times the volume of sample in Folch and four times Bligh and Dyer).

Sampling and sediment analysis

The metabolite content of a sediment sample depends on the activity of microorganisms in that habitat at the moment of collection. Samples must be collected and stored correctly to block any further metabolic activity.
Snap freezing with liquid nitrogen is the most efficient way of blocking all metabolic process and it is performed by putting the sample in liquid nitrogen (-195.8 C). To avoid contamination, pre-leaching of all plastics in sampling equipment and the use of pre-combusted amber glass bottles is recommended [27]. If the analysis is to be carried within 48 h it is also important to transfer the sample back to the laboratory as quickly as possible inside a chilled (4 °C), dark box to minimise the transport temperature. For storage, samples should be kept at -20 °C.

Experimental introduction

Venice Lagoon is suffering from deep changes in its environment, mostly due to human activities. These changes reflect on the organisms and microorganisms that inhabit it; understanding their functions and roles in the ecosystem will support lagoon restoration efforts. We aimed to characterize ten marsh sediment samples collected from the Venetian lagoon to understand the microorganism activity and the biological processes in that area. This understanding can be a crucial factor to preserve the ecosystem of the most famous lagoon in the world.
Sediments are some of the least investigated matrices, especially with NMR. A fast, accurate, and quantitative molecular characterization of sediment samples can be useful to track and understand ongoing biological processes.
In this study, we applied techniques first reported by Christina A. Fox et al. 2018 for

NMR sediment characterization. However, the reported spectra are of low quality, with broad and completely overlapped signals. The authors were only able to divide the spectra in regions that indicate approximately the type of chemical moiety, these results are in line with the reported molecular complexity of DOM [23].
In this study, we aim to better identify sediment metabolites by applying solvent extraction methods to NMR sample preparation, in order to obtain high-quality NMR spectra that can give more detailed information.

MATERIALS AND METHODS

Sediment sampling

Ten samples were collected in Venice, Burano and Ca' Smerghetto in the Venice lagoon in Italy on May 9th, 2023. Seven sediments and three water samples were collected in 50 mL falcon tubes, transported at 15 °C and stored at -20 °C.

Laboratory processing

Pore-water analysis

Two grams each of sample were put in Eppendorf tubes (10 mL) and kept in ice, to maintain low temperature. Wet weight and water content were measured by drying the samples in the oven at 40 °C for 2 days (Table 4).

Table 4: Sample characteristics

Sample	1	2	3	4	5	6	7	8	9	10
Weight(g)	1.9980	2.0360	2.0357	2.0065	2.0381	1.9843	2.0261	2.0103	1.9826	1.9808
pH	7.1	7.43	7.35	8.01	7.73	7.27	7.45	7.72	7.72	8.18
Water content (%)	66	59,43	38,57	5,53	73,20	34,69	31,48	95,65	98,82	99,27

Samples were centrifuged (Allegra X-30R Centrifuge, Beckman) at 7600 x g for 15 min at 3 °C.
A minimum of 400 µL of supernatant was transferred to Eppendorf tubes and kept in ice; pH was measured at this stage (Mettler Toledo - Table 4). Then, 100 µL of D_2O were added as well as 2 µL of 99% Trimethylsilylpropanoic acid (TSP). Samples were then centrifuged (7600 x g for 15 min at 3 °C), supernatants were then transferred in 5 mm NMR tubes (Figure 6).

Figure 6: NMR pore-water tubes

Folch extraction

Approximately 5 g of sediment were put into Teflon tubes and processed following the Folch extraction method [24].
After the addition of 1 mL of water and 3 mL of methanol, samples were vortexed (Vibromix) for 1 min and 6 mL of chloroform were further added.

Samples were then mixed for 15 min in a rotary shaker at 1820 rpm at room temperature, left in ice for 10 min to improve the phase separation and centrifuged at 7500 x g for 20 min at 3 °C. The two solvent phases were separated and transferred in glass vials.

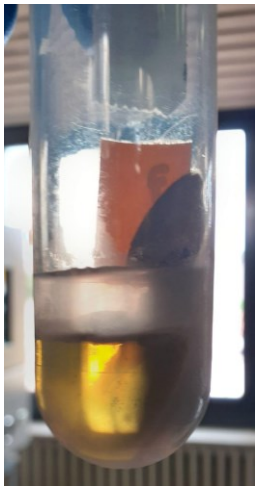


Figure 7: phase separation of sample n°7

Water-methanol and chloroform were clearly separated (top and bottom layer, respectively) as shown in Figure 7. A heavier layer of sediment was clearly observable at the bottom of the tube, as well as the layer of proteins, between the two solvents. While water-methanol extracts appeared clear, chloroform extracts were turbid and had to be filtered using a cellulose filter. Filters were prepared and checked for contamination using clean chloroform. Blank samples (chloroform only filtrate) were analysed along with other samples.

All chloroform vials and water-methanol vials from samples 1-4 were put in a smart evaporator, at 42 °C with N_2 flow, while the other six water-methanol vials were transferred to Teflon tubes and lyophilized due to time restrictions.

The solid residue in samples prepared with the smart evaporator were confined to the bottom of the vials while samples prepared with the lyophilizer appeared much more scattered due to the boiling of the liquid.

All chloroform extracts, including the blank, were suspended in 600 µL of deuterated chloroform and transferred into a 5 mm NMR tube.

The water-methanol extracts were suspended in 600 µL of D_2O with 3 µL of 99% TSP, then transferred into a 1.5 mL Eppendorf tube and centrifuged at 10000 x g for 10 min at 4 °C.

Supernatants were then carefully transferred into a 5 mm NMR tube.

NMR spectra acquisition and processing

NMR spectra were acquired with a Bruker Avance Neo 600 MHz spectrometer at the chemistry Department of the University of Padova. The spectrometer is equipped with a Prodigy cryoprobe. We acquired three 1D ^{1}H-NMR spectra for each sample. All extracts were acquired and processed using Topspin 4.2.0 software.

Pore water and D_2O spectra were acquired applying the noesypr1D pulse (Figure 8) while $CDCl_3$ spectra with zg pulse program (Figure 9), both with 4 dummy scans, 8 scans, spectral width of 11.9 kHz and receiver gain set to 101. Sample number 6 was further analysed through the acquisition of a 2D ^{1}H-^{1}H TOCSY NMR spectrum with 16 dummy scans and 8 scans. After acquisition, all spectra were processed performing manual phase correction and automatic baseline correction.

Peaks were initially identified by comparison with other metabolomics characterizations reported in the literature (Frizzo et al 2021) [28]. A more precise approach was then adopted, submitting known chemical shifts to public databases such as the Human Metabolome DataBase (HMDB) [29] or Guided Ideographic Spin System Model Optimization NMR database (GISSMO) [30], checking the peak relative intensity, multiplicity, and J coupling for each proton of each identified compound.

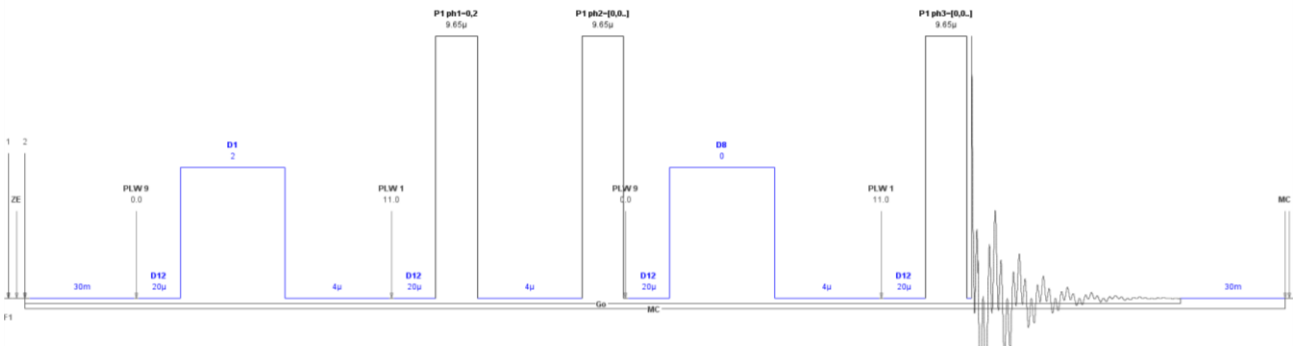


Figure 8: noesypr1D pulse program

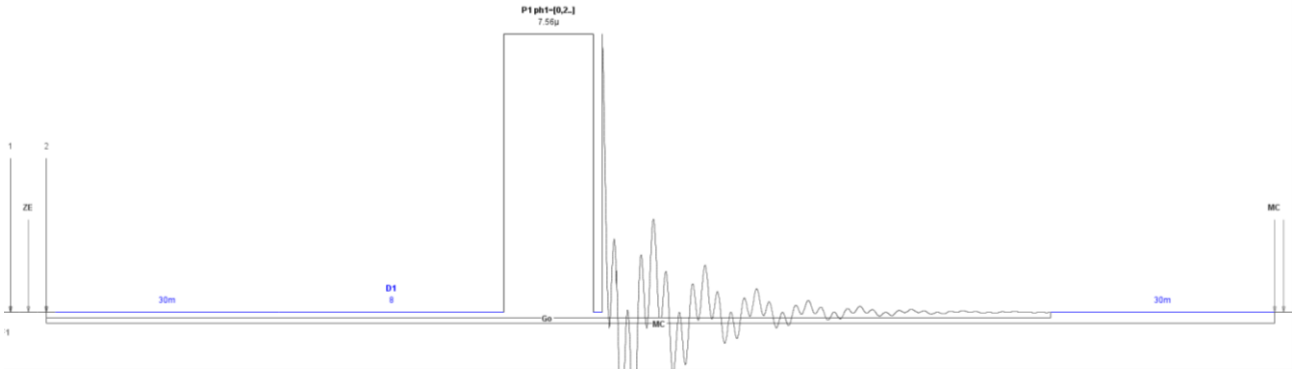


Figure 9: zg pulse program

RESULTS

Three 1D ^{1}H-NMR spectra were acquired for each sample, further adding a 2D ^{1}H-^{1}H TOCSY NMR spectra for sample 6 methanol extract. Sample 6 was then selected as representative due to signal quality, signal intensity and similarity to other spectra. Results are reported below.

Common spectral characteristics

NMR spectra were analysed qualitatively. Despite signal suppression (as part of noesypr1D pulse) the highest signal in pore water and methanol spectra comes from water (H_2O). Other peaks are clearly visible and correctly shaped with some overlaps in all spectra.

Pore water spectra showed abundant signals from sediment metabolites: it contains 118 peaks. The comparison with sample number 10, which is made up with more water than others and presents almost only the water peak is shown in figure 10. Most peaks (55, about 50% of the total) are located from 3 to 4 ppm; conversely the spectral region beyond 5.5 ppm is empty.

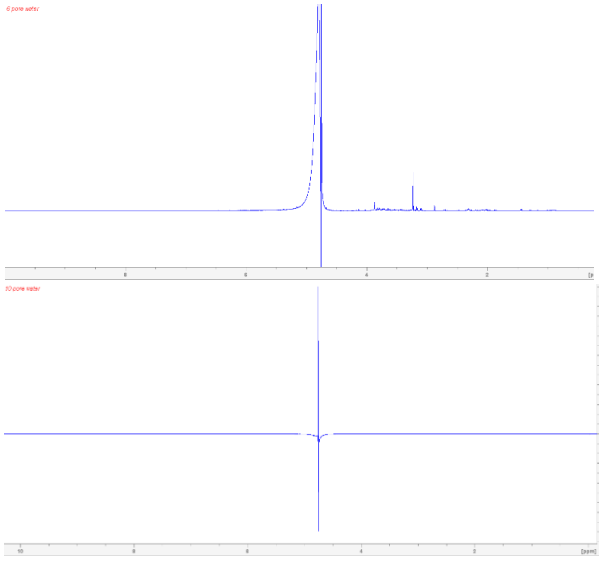


Figure 10: peak presence comparison between sample 6 (above) and 10 (below) pore-water spectra

The methanol spectrum contains 91 peaks and are mostly found between 3 and 4 ppm. In contrast with pore water spectra, 7 peaks appear in the aromatic region, probably due to higher concentration.
The pure chloroform spectrum shows 39 peaks, revealing a contamination of the solvent either from micropipette tips or from NMR tube caps. Consequently, the $CDCl_3$ spectrum of the extract shows in total 83 peaks, but it is difficult to discriminate between sample peaks and contamination peaks. Chloroform peaks are mostly located in the region between 0.5 to 2.5 ppm and beyond 6 ppm.
Given these results, we decided to consider mainly methanol extracts for signal assignment. Most signals are common to all the samples with some shifts due to pH variations. The reference sample of choice is number 6, the following considerations and assignments refer to spectra reported in Figure 11.

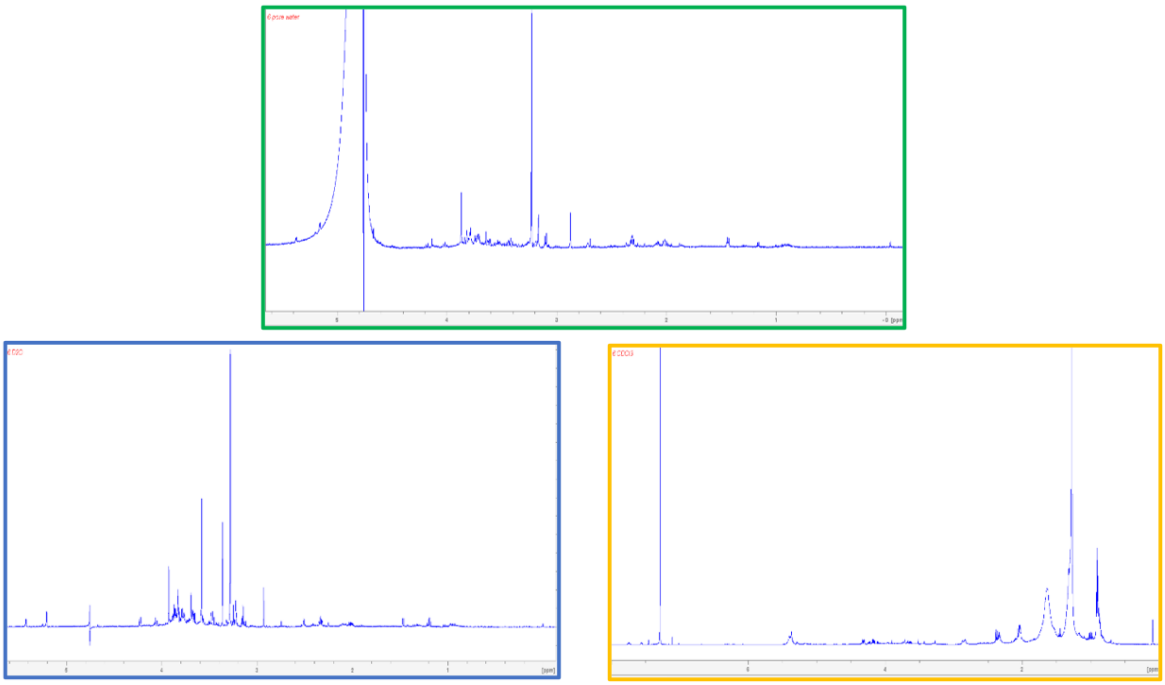


Figure 11: ^{1}H-NMR spectra of sample n°6: upward the pore-water one, bottom left the methanol extract and bottom right the chloroform exctract.

Metabolite assignment of sample n°6

A total of 40 metabolites were identified, 26 from the methanol extract and 14 from the chloroform extract.
In the methanol spectrum, most of the peaks were assigned using the TOCSY spectrum. Amino acid side chain display peaks from 0.93 to 2.12 ppm while in the region between 3.25 and 3.88 there is a massive presence of carbohydrate peaks.
In the chloroform spectrum, peak assignment was much more difficult because of the lack of 2D and the contamination of chloroform. However, we tried to identify some compounds assuming their presence and verifying their peaks in the chloroform spectra. In that case again, there is a region between 0.67 and 0.84 where cholesterol peaks falls and between 0.85 and 1.30 where fatty acid peaks fall.

Methanol extracts

Table 5: metabolites assignment from methanol extracts

Chemical shift	Multiplicity	Assignment	Chemical shift	Multiplicity	Assignment
0.9394	t	Isoleucine	3.3219	d	Unknown
0.9639	t	Leucine	3.3645	s	Proline
0.9820	d	Valine	3.417	t	Taurine
1.0106	d	Isoleucine	3.4531-3.4859	m	Cellobiose Gluconic acid Glucose
1.0382	d	Valine	3.503	t	Glucose
1.1972	q	3-Hydroxybutyric acid	3.5671	d	Threonine
1.3542	d	Threonine	3.5749	s	Taurine
1.4236	d	Unknown	3.5878	s	Glycine
1.4688	d	Alanine	3.6586	t	Glutamic acid
1.9963-2.1201	m	Proline Glutamine Glutamic Acid	3.6709	s	Sucrose
2.143	s	Acetylcholine	3.6766	d	Isoleucine
2.2549	s	Cellobiose	3.6945	s	Dimethylglycine
2.325	s	3-Hydroxybutyric acid	3.7135-3.7405	m	Gluconic acid Cellobiose
2.389	s	Succinic acid	3.772	t	Glutamine
2.34	t	Proline	3.8324	t	Ethanolamine
2.414	s	Glutamine	3.7839-3.8873	m	Gluconic acid Glucose Sucrose
2.519	s	Unknown	3.928	s	Glycolic acid
2.748	s	Unknown	4.0665	t	Sucrose
2.933	s	Dimethylglycine	4.2246	d	Sucrose
3.1205	s	Ethanolamine	4.6693	d	Unknown
3.1374	s	Malonic acid	5.2098	d	Glucose
3.1502-3.2246	m	Histidine	5.4283	d	Unknown
3.2281	s	Acetylcholine	5.8612	s	Unknown
3.2492	t	Glucose Taurine	7.3386	d	Unknown
3.284	s	Cysteic acid	8.4631	s	Formic acid

Chloroform extracts

Table 6: metabolites assignment from chloroform extracts

Chemical shift	Multiplicity	Assignment	Chemical shift	Multiplicity	Assignment
0.67	s	Cholesterol	2.6490	s	Acetyl CoA
0.800-0.841	m	Cholesterol	2.7970	t	Linoleic Acid
0.8570	t	Lauric acid Caproic acid	2.8830	t	Acetyl CoA
0.8800	t	Palmitic acid	3.2750	s	Acetyltributylcitrate
0.9040	t	Linoleic Acid	3.5188	s	Phenylacetic acid
1.0980	s	Cholesterol	3.9030	s	Vanillin
1.0130	s	Acetyl CoA	3.9800	s	Acetyl CoA
1.2770	s	Lauric acid Palmitic acid Myristic acid Stearic acid	4.0920	s	Acetyltributylcitrate
1.3030	m	Linoleic Acid	4.1630	s	Acetyltributylcitrate
1.6230	m	Linoleic Acid Lauric acid	5.3481	t	Linoleic Acid
2.0340	q	Linoleic Acid	5.392-5.414	m	Cholesterol
2.0610	s	Acetyltributylcitrate	6.1460	m	ATP
2.1568	t	Caproic Acid	6.2850	s	Vanillin
2.2430	t	Acetyl CoA	7.0289	s	Vanillin
2.3340	t	Linoleic Acid	7.7231-7.7592	m	Benzoic acid
2.3460	t	Palmitic acid	8.1119	s	ATP
2.3387	s	Pyruvic acid	8.2220	d	Benzoic acid
2.3449	t	Lauric Acid			

DISCUSSION

Free amino acids are the most abundant class in our sediment samples. Isoleucine, leucine, valine, threonine, alanine, proline, glutamine, glutamic acid, glycine, and taurine were identified as well as dimethylglycine, a glycine derivative.

Furthermore, cysteic acid, a cysteine derivative that is a precursor in the taurine metabolic pathway, was identified [31]. Those two are the only sulphonated compounds detected.

Carbohydrates are also very abundant and their peaks present stronger signals. Disaccharides such as cellobiose, and sucrose, but also glucose and its derivatives such as gluconic acid are present.

The carboxylic acids detected were succinic acid, formic acid, pyruvic acid, malonic acid and 3-hydroxybutyric acid.

Succinic acid is a dicarboxylic and is an important and ever-present metabolite. It participates in the citric acid cycle and AA biosynthesis (glutamate, histidine, tyrosine, proline, methionine, taurine, arginine, glutamic acid) and degradation (valine, leucine, isoleucine). It also plays a biological role in apoptosis. Formic acid is the simplest carboxylic acid and is involved in nucleotides de novo purine biosynthesis and glycine, serine and cholesterol metabolism. Pyruvic acid is an α-keto acid that is involved in every AA, fatty acids and carbohydrates metabolism. It is produced from glucose through glycolysis and provide energy to cells via the citric acid cycle.

Malonic acid and 3-hydroxybutyric acid come from fatty acid metabolism and may indicate a high presence of this metabolic pathway in these environments [32].

This evidence is further supported by the presence of acetylcholine and ethanolamine, both involved in the metabolic synthesis of phospholipids. Fatty acids, both saturated and unsaturated, such as lauric, palmitic, stearic, caproic, linoleic and myristic acids were detected along with cholesterol, and are probably constituents of cell membranes.

Aromatic compounds are less represented in our samples. Except for cholesterol, three metabolites were identified: vanillin, a lignin degradation product; phenylacetic acid and benzoic acid that also present a carboxylic group [15].

Acetyl CoA and ATP were also found; the first is involved in the citric acid cycle, AA and fatty acid metabolism and steroid biosynthesis, while the second is a nucleotide used by living organisms to transport energy.

A compound normally not present in this environment was detected: acetyl tributyl citrate (ATBC).

ATBC was born as a safer and more environmentally friendly alternative to phthalates in plastic products. Its concentration in compost and agricultural soils is not regulated by any European regulation and there are very few, if any, research studies on its presence in compost and soil. This is probably because ATBC is classified as a non-toxic additive [33]. However, recent findings indicate that long-term exposure to ATBC at environmentally relevant concentration (0.5 µg/L) caused a significant adverse effect on the reproductive system of adult zebrafish [34]. There are evidence indicating that ATBC might disrupt mouse antral follicle function and be detrimental to mouse ovarian function at low concentrations (10 mg/kg/day)

(Rasmussen et al., 2017) [35]. All these evidence suggests that more information is needed for ATBC risk assessment.
However, our single detection is not evidence of its presence in this environment, and we cannot quantify it by our spectrum [36].

CONCLUSIONS

We acquired 1D ^{1}H NMR and 2D ^{1}H-^{1}H TOCSY spectra of saltmarsh sediments to investigate the metabolic processes ongoing in this ecosystem.
In contrast to the spectra reported by Fox et al. 2018 [23], our spectra appear well defined with narrow peaks. These features allow us to distinguish 118 peaks for pore-water, 91 peaks for methanol extract and 83 peaks for chloroform extract and to identify a total of 40 metabolites, 26 from methanol extract and 14 from chloroform extract.
The two most abundant metabolites classes are amino acids and carbohydrates, but also fatty acids, carboxylic acids, lipids and aromatic compounds were detected.
The spectra can be improved by increasing the number of scans, reducing the signal to noise ratio. The contamination of blank $CDCl_3$ samples affects the detection of metabolites in all $CDCl_3$ spectra.
The results of this study indicate that a correct sampling, storage and processing protocol with an efficient extraction method, leads to a well-defined NMR spectrum. From this spectrum, many metabolites can be identified.
The abundance of metabolites in our samples, indicate a high organism activity in those environments and consequently highlights their crucial role in storing and processing CO_2 and organic matter.

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