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Beach-cast seagrass wracks: greenhouse gas emissions and energy recovy

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To Gabriele and Mattia

"The cure for anything is salt water:

sweat, tears or the sea."

Isak Dinesen (Karen Blixen)

Content

Abstract

Seagrass meadows are among the most productive ecosystems in marine environments. Like many terrestrial higher plants, marine seagrasses lose their old leaves during annual or inter-annual senescence, and a significant proportion of these residues is transported in surface waters and washed up on shores by surf, tides and winds. These beach-cast seagrass wracks provide important ecosystem services, such as reducing wave impact, protecting beaches from erosion, providing habitat to birds and invertebrate species that colonize shorelines, and being a primary food resource for beach detritivores. However, seagrass residues accumulation on beaches, following meadows degradation, can negatively impact tourism. Therefore, wrack piles are frequently collected and disposed of in landfills or biomass waste facilities, and the adoption of these management practices implies substantial environmental and economic costs. On the other hand, wrack piles might be a significant source of greenhouse emissions (GHGs). Recent studies reported CO_2 and CH_4 emission rates and suggested possible mitigation options, such as energy conversion and biochar production through pyrolysis. Even though quantitative estimates of both seagrass coastal distribution and residues disposal to seashores are partially available, at least at regional level, the assessment of their contribution to global GHGs emissions is still lacking, due to a knowledge gap about the effects of peculiar beach ecosystems environmental conditions on seagrass decay rates. Moreover, many studies have proposed several reuse options of beached seagrass residues in order to reduce both economic costs of collection from the shoreline and disposal in landfills and to offer a more sustainable beaches management. seagrass biomass use for energy production is under consideration in several countries of the world, as it combines the continuous

increase in energy demand, sustainable costs of applied technology and social acceptance.

In this research, the seagrass wracks decomposition dynamics were investigated in both controlled conditions and experimental fields in North-East Italy, with focus on CO_2 and CH_4 emissions, as a function of temperature, salinity, water supply. Moreover, the problems and perspectives concerning the assessment of beach-cast wrack contribution to the global GHGs emissions were highlighted. Using obtained results, the research then focused on energy recovery of beached seagrass litter as biomass source for anaerobic digestion. It was determined the potential methane production, the average biogas yields using different relative concentrations of seagrass biomass and sewage sludge and salinity effect on anaerobic digestion. Moreover, through genetic analysis, salinity and temperature effect on the anaerobic bacterial community composition was highlighted and the most relevant microbial families for biogas production were determined.

1. Introduction

1.1. State of the art

Humans depend on marine ecosystems for important and valuable services; however, their activity have also altered these habitats through direct and indirect impacts (Myers and Worm, 2003; Lotze et al., 2006; Halpern et al., 2015). As reported by several studies (e.g. Pimentel et al., 2010; Adeleke, 2017) human population continue to grow and migrate to the coasts, engendering a global increase of individual and cumulative pressures on oceans and sea spaces and on marine resources (Crowder et al., 2006). The runoff of pollutants and nutrients from lands into coastal waters, the destroy of natural habitats, as seagrass meadows, and the change of species composition are just some of the consequences generated by anthropogenic overexploitation of marine habitats (Short et al., 2007; Ferrari et al., 2016; Gilbert et al., 2018). Sea ecosystems have been increasingly altered worldwide by a diversity of global, regional and local anthropogenic stressors (Corrales et al., 2018). Since several decades, scientific community increased knowledge about the impacts of single stressors and their cumulative effect on marine habitats, offering possible management solutions that appear more sustainable (Caro et al., 2018; Furlan et al., 2018; Mackenzie et al., 2018). One of the objectives of world administrations is the reduction of marine non-renewable resources exploitation by proposing new technological uses of sustainable resources (Marquez et al., 2016; Basanti and Gualtieri, 2018; Kiran et al., 2018). Indeed, it is possible to decrease the anthropic impacts using this operative approach, with a reduction in greenhouse gases release to the atmosphere (Seghetta and Goglio, 2019). Furthermore, understanding alterations engendered by human activities and diminishing anthropic impacts is fundamental in order to reduce biological changes in the structure and functioning of marine ecosystems associated with changing climates (Hoegh-Guldberg et al., 2019; Lotze et al., 2019).

1.1.1. Seagrass biology

Marine seagrasses are flowering plants (*Magnoliophyta*) consisting of roots, stem, leaves, that include 72 species who live completely submerged and develop along coasts around the world (Larkum et al. 2006). Seagrasses were terrestrial plants that have returned to the sea between 100 and 120 million years ago, in Cretaceous (Larkum and Den Hartog, 198). Seagrass belong to very limited number of plant families, classified as *Alismatiflorae* (*Monocotyledonae*), and they are: *Cymodoceaceae*, *Hydrocharitaceae*, *Posidoniaceae* and *Zosteraceae* (Dahlgren et al., 1985). In Mediterranean Sea only five species are present and specifically are: *Posidonia oceanica*, *Cymodocea nodosa*, *Nanozostera noltii*, *Zostera marina* and *Halophila stipulacea* (Procaccini et al., 2003; Figure 1). Only *P. oceanica* is endemic of Mediterranean Sea, whereas *H. stipulcea* comes from the Red Sea (Gambi et al., 2009).

P. oceanica (L.) Delile is characterized by nastriform leaves, up to 50 cm long, and present a developed rooting apparatus that can penetrate the sediment even at remarkable depth (Bianchi and Buia, 2008). The plant has creeping or erect stems, called rhizomes: they can be orthotropic, with vertical growth, or plagiotropic, with horizontal growth, and their morphology depends on the available space for growth (Caye, 1980). Thanks to this alternation of growth phases, a network of rhizomes, root and sediment is generated which gives rise to *matte*: it is a persistent elevated structure of considerable ecological importance (Moliner and Picard, 1952). The rise of the *matte* due the continuous meadow growth, is generally estimated between 10 cm/century (Boudouresque and de Grissac, 1983) and 18 cm/century (Mateo et al., 1997), even if an increase of *matte* up to 1 m/century was reported elsewhere (Moliner and Picard, 1952). The rhizomes end with bundles composed by 4-8 leaves, which live for 5 to 8 months (Pergent-Martini and Pergent, 1994). The plant has a hydrophilic pollination and the fruit is a fleshy tough drupe, called sea olive. However, *P. oceanica* reproduces mainly by vegetative way (Boudouresque et al., 2012).

C. nodosa (Ucria) Asch. has nastriform leaves, up to 30 cm long, with a dentate and round leaf apex, a short vertical rhizome that ends with leaves-bundles and ramified roots (Perez et al., 1994). The fall of foliar bases leaves a circular scar, called knot, on vertical rhizomes (Caye and Meinesz, 1985). *C. nodosa* is the second seagrass in Mediterranean Sea for distribution, showing a wide environmental tolerance (Duarte and Sand-Jensen 1990). It forms meadows that are mono-specific or mixed with *Z. noltii* (Mazzella et al., 1993) and it is considered a pioneer species which can quickly colonize bare areas of the sea floor (Borum and Greve, 2004).

Z. marina (L.) is the second largest specie of seagrass in Mediterranean Sea (Buia and Giaccone, 2008). The plant present nastriform leaves up to 1 m long (Procaccini et al., 2003). It has a cosmopolitan distribution and although is an infralittoral specie, it tolerates long surfacing periods (Moore and Short, 2006). Moreover, it prefers sandy and muddy bottoms with moderate hydro-dynamism (Widdows et al., 2008).

N. noltii Hornem. has smaller dimensions than the other species, with nastriform and thin leaves about 30 cm long (Pérez-Lloréns and Niell, 1993). It is a cosmopolitan seagrass and favours very shallow coastal environments, estuaries and lagoons (Peralta et al., 2005).

H. stipulacea (Forssk.) Asch. is a small seagrass with elliptic leaves and has a tropical-subtropical origin: it was introduced in the Mediterranean Sea through the Suez Channel and currently its distribution is widening (Procaccini et al., 1999).

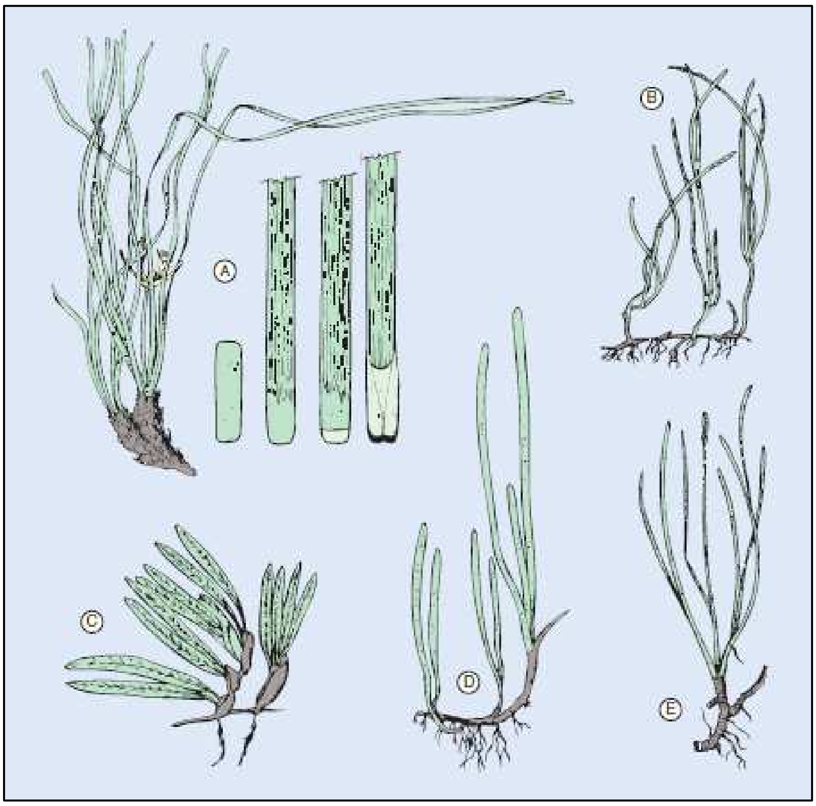

Figure1: Seagrasses in Mediterranean Sea. A. *Posidonia oceanica*; B. *Cymodocea nodosa*; C. *Halophyla stipulacea*; D. *Nanozostera noltii*; E. *Zostera Marina*.
(in: Buia and Giaccone, 2008)

1.1.2. Seagrass ecosystem

Seagrasses develop extensive meadows at the interface between water column and sediment in tidal or subtidal environments (Marbà et al., 2007). Normally seagrass meadows extend from the surface to 40-45 m depth, if physico-chemical conditions of see bottom and water column favour their colonization (Koch, 2001; Koch and Verduin, 2001; Gobert et al., 2006; Boudouresque et al., 2012). Seagrass meadows are protected in Europe as prior natural habitats in the Directive 92/43/EEC Annex I. Especially *Posidonia oceanica* meadows are included in the Sites of Community Interest (SCIs), for which special plans of management and conservation must be designnated (EEC, 1992).

Meadows are characterised by an upper and lower limit. The upper limit is the shallowest depth reached by meadow and depends mostly by slope of sea bottom and by hydro-dynamism (Boudouresque et al., 2012; Vacchi et al., 2010, 2014). Indeed, in favourable conditions, the most relevant feature of the upper limit is the occurrence of extremely shallow formations in calm water, with leaves reaching and spreading out on the sea surface (Koch et al., 2006). The lower limit is the greatest depth extend by the plant: it depends on the amount of light reaching the seafloor, on water column transparency and on local geomorphology, currents and wave exposure (Short et al., 2002; Ryan et al., 2007; Infantes et al., 2009; Vacchi et al. 2012). The morphology of the limits is a valid local bioindicator, especially the presence of regression in the upper limit. Indeed, they provide information about the factors that determine it, the health of the meadow, the hydrodynamic regime and the sedimentary balance, as well as the presence of anthropogenic factors (Duarte, 1991). For example, as shown by Montefalcone et al. (2010), regressive aspects of the upper limit are often correlated with meadow fragmentation, mostly resulting from human impacts. Seagrass meadows are also bioindicators on a global scale, because they are widespread and sensitive to environmental changes (Bhattacharya et al., 2003) and are able to integrate ecological conditions and processes over various timescales from weeks to millennia (Madden et al., 2009, López-Sáez et al., 2009; Martinez-Crego et al., 2010; Pergent et al., 2014; Leiva-Dueñas, 2019).

Indeed, the state of conservation of seagrass meadows represented an efficient tool for the assessment of government policy regarding actions undertaken to improve environmental quality (Pergent et al., 2005).

Seagrass meadows architecture can exhibit normally continuous cover of the seabed, however, *P. oceanica* meadows could be also organized in patches of various shapes, including strips parallel to the shoreline or cordons perpendicular to the shoreline (Borg et al., 2005; Boudouresque et al., 2012).

Meadows are considered as ecosystem engineers because they can modify the abiotic environment. Indeed, the canopy of the meadows reduces hydrodynamic energy from currents and waves, alter chemical and physical sediment characteristics, stabilize sediment and increase macrobenthic organisms abundance and biodiversity (Gacia and Duarte, 2001; Boudouresque et al., 2012; Brun et al., 2009). Moreover, several studies documented sediment erosion following seagrass losses and erosion prevention by seagrass presence.

Most seagrass species colonise soft bottoms in wave-sheltered area, however *P. oceanica* may colonize sand, *matte* and rock, the last only in presence of an adequate amount of organic matter (Green and Short, 2003; Montefalcone et al., 2016).

Several papers demonstrated that the substratum influenced plant morphology and meadows characteristics (Badalamenti et al., 2015; Balestri et al., 2015;

Montefalcone et al., 2016; Vacchi et al., 2017). Indeed, when *P. oceanica* colonizes rocky bottoms, the plant decreases shoot size and increases density compared to soft substrata (Short, 1983; Giovannetti et al., 2008). Sedimentological features of the bottom are also known to control meadow development; however, their influence is less important in determining meadow characteristics, especially in shallow waters (Ryan et al., 2007). On the contrary, sediment features play a fundamental role in carbon storage (Dahl et al., 2016). Local geomorphology, currents and wave exposure play a very significant role in controlling the morphology and bathymetrical distribution of seagrass meadows (Infantes et al., 2009). Indeed, several studies shown that wave breaking represents the major constraint for the landward development of the meadows occurring on sedimentary beds, reducing the cover of living seagrass plants (Vacchi et al., 2010, 2012, 2014).

1.1.3. Seagrass distribution

Seagrass meadows cover about 0.1-0.2% of the global ocean (Fourqurean et al., 2012). However, nowadays globally 15% of seagrass species are threatened (Short et al., 2011) and meadows have declined worldwide (Waycott et al., 2009). In general, seagrass species develop in temperate or tropical regions: the north temperate region is dominated by species in the genus *Zostera*, while in the south temperate region species of *Posidonia* prevail (Short and Coles, 2001). Short et al. (2007) studied global distribution of seagrasses dividing the World in six geographic bioregions based on assemblages of taxonomic groups in temperate and tropical areas and the physical separation of the world's oceans. The obtained results are reported in table 1. The Tropical Indo-Pacific is globally the bioregion of highest species diversity (24 species), with a major diversity in Southeast Asia and north tropical Australia.

Table 1: The global distribution of seagrass species within 6 geographic bioregions based on assemblages of taxonomic groups in temperate and tropical areas and the physical separation of the world's oceans (from Short et al., 2007).
The boundaries of each bioregion are described, and the seagrass species of each bioregion are presented alphabetically, followed by seagrass species that have their centre of distribution in an adjacent bioregion or are invasive to a bioregion, designated with a "+". Species listed in brackets are conspecific with the preceding species. Species listed in parentheses require further genetic and morphometric investigation and may be conspecific with the preceding species.

Bioregion	**Description**	**Species**
1. Temperate North Atlantic (North Carolina, USA to Portugal)	Low diversity temperate seagrasses (5 species) primarily in estuaries and lagoons	*Ruppia maritima, Zostera marina, Zostera noltii, Cymodocea nodosa+, Halodule wrightii+*
2. Tropical Atlantic (including the Caribbean Sea, Gulf of Mexico, Bermuda, the Bahamas, and both tropical coasts of the Atlantic)	High diversity tropical seagrasses (10 species) growing on back reefs and shallow banks in clear water.	*Halodule beaudettei, H. wrightii (H. bermudensis, H. emarginata), Halophila baillonii, Halophila decipiens, Halophila engelmanni, Halophila johnsonii, R. maritima, Syringodium filiforme, Thalassia testudinum, Halophila stipulacea+*
3. Mediterranean (including the Mediterranean Sea, the Black, Caspian and Aral Seas and northwest Africa)	Vast deep meadows of moderate diversity and a temperate/tropical mix of seagrasses (9 species) growing in clear water.	*C. nodosa, Posidonia oceanica, Ruppia cirrhosa, R. maritima, Z. marina, Z. noltii, H. wrightii+, H. decipiens+, H. stipulacea+*
4. Temperate North Pacific (Korea to Baja, Mexico)	High diversity of temperate seagrasses (15 species) in estuaries,	*Phyllospadix iwatensis, Phyllospadix japonicus,*

	lagoons and coastal surf zones.	*Phyllospadix scouleri, Phyllospadix serrulatus, Phyllospadix torreyi, R. maritima, Zostera asiatica, Zostera caespitosa, Zostera caulescens, Zostera japonica, Z. marina, H. wrightii+, H. decipiens+, Halophila euphlebia+, Halophila ovalis+*
5. Tropical Indo-Pacific (East Africa, south Asia and tropical Australia to the eastern Pacific)	Largest and highest diversity bioregion; tropical seagrasses (24 species) predominantly on reef flats but also in deep waters, many commonly grazed by mega-herbivores.	*Cymodocea angustata, Cymodocea rotundata, Cymodocea serrulata, Enhalus acoroides, Halodule pinifolia, Halodule uninervis, H. wrightii, Halophila beccarii, Halophila capricorni, H. decipiens, Halophila hawaiiana, Halophila minor, H. ovalis, Halophila ovata, Halophila spinulosa, H. stipulacea, Halophila tricostata, R. maritima, Syringodium isoetifolium, Thalassia hemprichii, Thalassodendron ciliatum, Zostera capensis+, Z. japonica+, Zostera muelleri+ [Zostera capricorni]*
6. Temperate Southern Oceans (New Zealand and temperate Australia, South America, and South Africa)	Extensive meadows of low-to-high diversity temperate seagrasses (18 species) often growing under extreme conditions.	*Amphibolis antarctica, Amphibolis griffithii, Halophila australis, Posidonia angustifolia, Posidonia australis, Posidonia ostenfeldii complex, Posidonia sinuosa, R. maritima, Ruppia megacarpa, Ruppia tuberosa, Thalassodendron pachyrhizum, Z. capensis,*

		Z. muelleri [Z. capricorni], Zostera tasmanica [Heterozostera tasmanica], H. decipiens+, H. ovalis+, S. isoetifolium+, T. ciliatum+

Focusing in Mediterranean bioregion, *P. oceanica* and *C. nodosa* are the most abundant species (Procaccini et al., 2003). Moreover, *P. oceanica* colonizes seabottom areas to greater depths, while *Z. noltii* and *C. nodosa* occupy fairy intertidal and subtidal zones (Short et al., 2007). Telesca et al. (2015) reported that the extension of *P. oceanica* meadows was about 12,247 km², with a greater distribution in the eastern part of the basin (7,140 km²). Moreover, the seagrass was found to be present along 11,907 linear km out of a total coastline extension over 46,000 linear km. However, for about half of total linear coastline there was no information on seagrass presence or absence. *P. oceanica* was absent in the extreme south-east of the basin, probably due the excessive summertime water temperatures (Celebi et al., 2006). As reported in the study, *P. oceanica* meadows presented the maximum extension in Tunisia, Italy and Spain with a uniform distribution along the continental coastline and islands (Telesca et al., 2015). In Italy meadows extended in Tyrrhenian, Ionian Sea, and South-Western Adriatic Sea, with the exception of the main river mouths (Boudouresque et al., 2012).

C. nodosa is a seagrass distributed across the Mediterranean Sea, particularly in the eastern part of the Oriental Basin, and some locations in the north Atlantic, from southern Portugal and Spain to Senegal, including the Canary Islands and Madeira (Borum and Greve, 2004; Chefaoui et al., 2016). In several sectors of the Mediterranean, *C. nodosa* has taken advantage of the regression of *P. oceanica* to extend its own development (Montefalcone et al., 2007).

Zostera marina is the most widely distributed species, from the Atlantic Ocean to the Pacific Ocean, and from temperate regions to the Arctic Circle (Green and Short, 2003). In Mediterranean Sea, *Z. marina* meadows extend in open coasts in France, Italy (particularly in Adriatic Sea), Spain and coastal lagoons of the western Basin (Laugier et al., 1999). Moreover, the plant dominated the Black and Azov Seas (Telesca et al., 2015).

In Mediterranean Sea, *Zostera noltii* is confined to coastal lagoon in sheltered bays along coasts of the Basin northern part,where is often associated with *Z. marina* or *C. nodosa*, with which it may form mixed meadows (Short et al., 2011). Furthermore, the seagrass is dominating in Caspian Sea (Short et al., 2007).

1.1.4. Seagrass meadows role in carbon storage

Seagrass meadows are estimated to bury from 27.4 Tg C yr^{-1} to 50 Tg C yr^{-1} (Nelleman et al., 2009; Fourqurean et al., 2012), about 10% of annual organic carbon buried in the oceans (Duarte et al., 2005). In fact, carbon sequestered in meadows remains trapped for very long periods of time (centuries to millennia) resulting in very large carbon stocks (Duarte et al. 2005; Lo Iacono et al. 2008). As reported by McLeod et al. (2011), seagrass meadows bury carbon at a rate that is 35 x faster than tropical rainforests, and their sediments never become saturated. Most of thecarbon is stored in the soil: roots and rhizomes, that grow in anoxic sediment, have very low N and P relative to C content and therefore bacteria community decomposes very slowly (Fourqurean and Schrlau, 2003). Consequently, organic matter remains intact for thousands of years (Macreadie et al., 2014).

Meadows are able to accumulate considerable amounts of biomass and develop organic-rich soils composed of both autochthonous and allochthonous organic carbon (O.C.) (Kennedy et al., 2010). The autochthonous carbon is carbon removed by the atmosphere through photosynthesis and converted for use by plant tissue, especially roots; allochthonous carbon is carbon present in sediment and associated organic carbon from adjacent ecosystems, transported by waves, tides, and coastal currents and trapped by canopy (Mateo and Romero, 1996; Simeone, 2008). Recent studies confirmed that in seagrass meadows, an estimated 50% of carbon stored in soils is allochthonous (Kennedy et al., 2010; Boudouresque et al., 2012; Duarte et al., 2013; Dahl et al., 2016).

Meadows capacity to store carbon depends on species composition, meadow health status and architecture and habitat characteristics (Gruber and Kemp, 2010; Lavery et al., 2013; Rozaimi et al., 2013). As reported by Lavery et al. (2013), larger seagrasses, such as *P. australis* and *P. oceanica*, that have deeper and larger rhizomes, generate more refractory forms of structural carbon than simpler species, producing more labile form of carbon.

Moreover, the longer canopy of larger seagrass species reduces near-bottom water velocities by the frictional effects of vegetation (Peterson et al., 2004), reducing the resuspension of particles within the canopy and trapping the organic and inorganic material (Gacia and Duarte, 2001). However, seagrass patchiness, caused by anthropogenic or natural factors, affects hydraulics and consequently canopy capacity of reducing the suspended particulate matter (Folkard, 2005). As mentioned before, the meadow architecture influences carbon storage in seagrass sediment: a high canopy and a remarkable shoot density in the meadow have the ability to stabilize the sediment and thus potentially increase organic matter sedimentation (Fonseca and Cahalan, 1992; Hendriks et al., 2009). Considering sediment properties, grain size, porosity and density are important factors that affect oxygen distribution in the sediment and microbial community capacity to decompose the organic matter and thus influencing the carbon sequestration process (Dahl et al., 2016; Röhr et al., 2016; Serrano et al., 2016; Belshe et al., 2017). For example, as demonstrated by Dahl et al. (2016), high content of fine grain size, high sediment porosity and low sediment density are positively correlated with sedimentary organic carbon.

1.1.5. Seagrass ecosystem services

Seagrass meadows perform several functions in the ecosystems: direct and indirect contributions of ecosystems to human wellbeing are defined as ecosystem services (Boyd and Banzhaf, 2007; TEEB, 2010; Braat and de Groot, 2012). Several studies analysed ecosystem services produces by seagrass meadows, that are listed below:

- source of food for coastal and marine food webs (especially finfish, shellfish and mega herbivores including green turtles and dugongs) and support commercially and recreationally important fisheries species. Moreover, algal epiphytes, that grow on seagrass canopy, are highly palatable for small grazers (Connolly, 1994; Valentine et al., 2002);
- coastal waters oxygenation through photosynthetic activity; for example, Alcoverro et al. (1998) demonstrated that at 10 m depth meadow can generate till 14 l of oxygen per day; this considerable production is due to both leaves and epiphytes biomasses, especially in shallow waters;

- carbon sequestration from the atmosphere as shown by several studies, seagrass meadows are still strong CO_2 sinks as demonstrated by the comparison of carbon (PIC and POC) stocks between vegetated and adjacent un-vegetated sediments (Lo Iocono, 2008; Duarte et al., 2010; Mazarrasa et al., 2015);
- organic carbon export to adjacent ecosystems, as sublittoral reef or sandy habitats and sublittoral caves (Dimech et al., 2006; Cresson et al., 2012). Moreover, organic carbon is exported also on circalittoral and bathyal zones (Fourt and Goujard, 2012);
- sediment stabilization: indeed, meadows forms an obstacle to sediments movement on the bottom (Brunel and Sabatier, 2009) and trap fine particles, playing an active role in the sedimentary balance of the beach (Basterretxea et al., 2004);
- prevention of sediment resuspension due to the slow flow of canopy, that shelters underlying sediment (Folkard, 2005); it is influenced by the meadow geometry (Chen et al., 2007), shoot density (Gacia et al., 1999) and by vertical development of the canopy and roots growth (Pergent-Martini et al., 2006);
- improvement of water transparency and, thus, increasing light penetration, which supports further seagrass growth (Carr et al., 2010);
- wave attenuation: as reported by Gambi et al. (1989) and Gacia and Duarte (2001) hydrodynamic forces are reduced from 10% to 75% under the leaves and of 20% few centimetres above the meadow;
- shoreline protection: wave attenuation reduces littoral erosion and seagrass meadow regression increases wave effect on the shoreline, as reported by several studies (Pergent and Kempf, 1993; Pasqualini et al., 2000);

- habitat for microbes, invertebrates and vertebrates, often endangered or commercially important; in particular seagrass meadows are nursery areas for fishes and invertebrates (Hughes et al., 2009; Whitfield et al., 2017);
- trapping and cycling of nutrients: meadows play an important part in cycling coastal nutrients, accelerating nitrogen fixation and increasing diffusive nutrient flux to local waters (Short, 1987).

In the last decades, several studies applied different methods to economically evaluate the ecosystem services engendered by seagrass presence (Cullen-Unsworth and Unsworth, 2013; Vassallo et al., 2013; Ruiz-Frau et al., 2017). This analysis is useful mainly to determine the economic loss in terms of hectares of meadows lost due to intense human activities. For example, applying an energy valuation, Vassallo et al. (2013) estimated that a square meter of seagrass meadow in Bergeggi Island (Ligurian Sea) has a value of 172 € m^{-2} y^{-1} based on calculation of resources employed by nature. Moreover, considering the decrease of the extension, the monetary value of Bergeggi meadow decreased from 45 millions of € in 1990 to 24 millions of € in 2006, with an estimated loss of approximately 174 millions of € of natural capital in fifteen years. Furthermore, analysing bibliographic data, Campagne et al. (2015) estimated that a square meter of seagrass meadow in Mediterranean Sea in France presents an economic value ranging from 28,500 and 51,500 € m^{-2} y^{-1}, with an annual loss ranging between 1.11 and 2.00 * 10^6 €, due the decline of *P. oceanica*.

1.1.6. Natural and human impacts on seagrass meadows and consequences

Seagrass ecosystems are among the most threatened ecosystems on Earth, with an increased global loss from 0.9% y^{-1} in the 1940s to 7% y^{-1} toward the end of 20th century: almost 29% of the areal extent of seagrasses has disappeared globally since 1879, implying that 1/3 of goods and services they provide has been already lost (Waycott et al., 2009). Since the last decades, rapid global losses are attributable to anthropogenic impacts, especially due the accelerated and uncontrol coastal development (De los Santos et al., 2010). As mentioned before, the loss and the degradation of seagrass meadows reduce important ecosystem services they provide (Orth et al., 2006).

Natural factors that influence meadows development are mostly physico-chemical parameters: temperature, salinity, waves, currents, turbulence and light (Koch and Verduin, 2001). Temperature and salinity generate a direct impact on seagrass growth and distribution (Masini and Manning, 1997), while the hydrodynamic conditions are essential for seeds survival and engraftment, for plant stability and for the pollination (Koch et al., 2006). Moreover, storms can uproot rhizomes and sediment resuspension, causing a decrease of light availability and a burial of leaves wraps, that can lead to plant depth (Bell et al., 2010). Several studies have analysed the interaction between hydrodynamism and seagrass meadows geometry (Schanz et al., 2002; Infantes et al., 2009; Vacchi et al., 2017; Meysick et al., 2019). In general, seagrass meadows are more fragmented in bottom areas that are more influenced by local hydrodynamism (e.g. rip currents) than areas more protected by waves (Infantes, 2009). Moreover, the interaction between hydrodynamism and bottom surface directly controls the position of meadow upper and lower limit (Vacchi et al., 2010, 2012).

Human impacts affecting seagrass ecosystems are both direct and indirect: they can influence meadows locally but also far away from the disturbance sources (Duarte, 2002; Boudouresque et al., 2009). Often there is a synergy among impacts, which are not always easily separated (Boudouresque et al., 2009) As reported by Duarte (2002) direct impacts are mechanical damage (caused especially by anchoring, dredging and trawling), water and soil eutrophication, salinity increase or decrease (due the presence of water desalinisation systems or spillage of hypersaline substances), shoreline urbanisation (e.g. dams, ports, embankments, coastal protective structures, cable and pipes laying, new beach resorts construction), land reclamation, presence of fisheries installations, introduction of allochthonous species (especially herbivores and plants seagrass competitors) and siltation change (e.g. by outfalls, increased run-off, dredging/disposal of dredge spoil, beach nourishments). Indirect impacts include the effects of global anthropogenic changes, as seawater temperature increase and sea level rise, increase GHGs concentration in atmosphere and in oceans (especially CO_2 and CH_4), increase in intensity and frequency of wave action and storms and food web alterations (Short et al., 2011).

In response to the global seagrass crisis, there is a greater need to increase the conservative management of seagrass meadows and their associated ecosystem services, implement shared policies among Countries, increase protected areas and educate for the public and resources management (Kenworthy et al., 2006; Orth et al., 2006).

1.1.7. Banquettes: ecology and management

As terrestrial plant, seagrass sheds their annual or interannual senescent leaves: a part of non-consumed leaves is accumulated within the meadow, where there is the basis of the detritus food web; other remarkable part of necromass is exported out of the meadow (Boudouresque et al., 2012). The Exported rate ranges between 10 and 55% of the total primary production (Mateo et al., 2003; Boudouresque et al., 2012); however, in some meadows the exported necromass was estimated up to 95% of annual leaf production (Ochieng and Erftemaijer, 1999). The senescent leaves are transported towards sublittoral habitats or deep waters or toward beaches by surf, tides and wind (Boudouresque et al., 2015). Along the coastline, seagrass leaves constitute a significant proportion of wrack (Machas et al., 2006), engendering stockpiles, called banquettes, that can even reach considerable dimensions (Boudouresque et al., 2015). Seagrass litter can remain on the beach even for several weeks and can be degraded on site by decomposers or return to the sea in presence of stormy weather or speed current (Machas et al., 2006; Cantesano, 2010). Seagrass necromass can be transported by winds from the beach up to the dune and foredune, also for several hundred meters inshore (Cardorna and Gacià, 2008) and their presence favours the growth of halophilic vegetation (ISPRA, 2010).

Seagrass wracks have an important ecological value: they are the basis of the beach fauna’s food chain, represent a habitat for a number of marine species, provide protection to coastal dunes (Nordstrom et al., 2011) and represent a protection for the shoreline, especially during the occurrence of intense storms (Mateo et al., 2003). Indeed, seeds, roots and plant fragments, many of which are marine autochthonous species, take up residence and germinate in the presence of seagrass wracks because they create conditions of “microfertility” (Jackson et al., 2002;

Simeone and De Falco, 2012). Moreover, seagrass wracks play a role in the exchange of material between beach and foredune (Nordstrom et al., 2011). For example, field observations in Sardinia have shown that, in some cases, foredune systems are constituted of alternating layers of *P. oceanica* leaves and sediment (De Falco et al., 2003).

Seagrass stockpiles are a significant sink of carbon and other important nutrients: as reported by Mateo et al. (2003), carbon, nitrogen and phosphorous in *Posidonia oceanica* banquettes represented the 71.0, 27.2 and 8.7% of meadow leaf production, respectively. Moreover, the element concentration increases exponentially along the top 150 cm and decreases below this level (Mateo et al., 2003). Rodriguez-Echeverria et al. (2009) showed that high microbial activity in seagrass wracks can reduce the substrate pH values (reaching neutral pH) through the addition of organic matter. Moreover, the substrate pH is a further factor that controls nutrient assimilation; in particular, phosphorus uptake is known to be favoured in neutral conditions (Marschner, 1995; Del Vecchio et al., 2013).

The presence of banquettes and their dimension is closely related to wind and sea conditions. Indeed, banquettes are a result of a dynamic process of accretion and destruction: during Winter, banquettes present the maximum heights due the intense storms and winds; however, they collapse due the erosion of their base by wave action, as reported in Figure 2 (Mateo et al., 2003; Simeone et al., 2013).

This accretion and destruction process represent a protection for the beach especially during the Winter season: their presence directly interacts with waves, reducing their intensity (De Falco et al., 2006; Nordstrom and Jackson, 2012). Moreover, several studies showed the significant role of banquettes in the shore stability because they modify the beach profile and reduce sediment movement, trapping hight amount of sand (Chessa et al., 2000; Simeone and De Falco, 2012; Vacchi et al., 2017). However, excessive accumulations of seagrass wracks close to urban centres can negatively impact tourism. Indeed, the presence of seagrass litter is interpreted by tourists as unhealthy conditions of shoreline and as a poor beach aesthetic value (Corraini et al., 2018). So, the lack of beach cleanliness can lead to a loss of recreational potential of a beach which negatively impacts the economy and social well-being (Portman and Brennan, 2017). Moreover, seagrass wracks represent a source of odours during decomposition process and fires can generate from stockpiles, especially during Summer, with consequent problems with public health and safety (Parente et al., 2013).

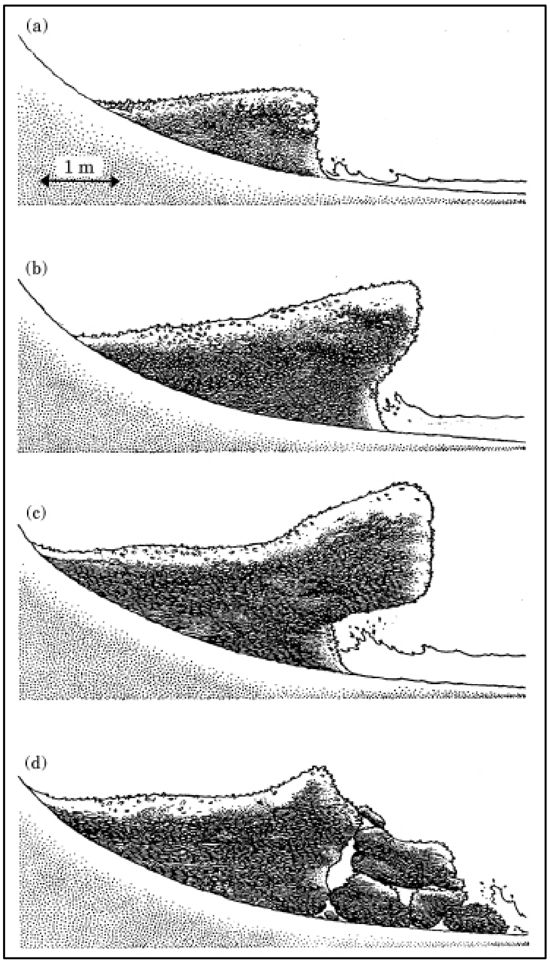

Figure 2: (a) Initial stage, (b) gain in size, (c) maximum height, (d) collapse of banquette. (From Mateo et al. 2003)

It is reported by many studies that faecal indicator organisms are able to persist, and possibly proliferate, in supra-littoral wrack piles on a beach that holds the sand moist (e.g., Ward, 2009; Dunhill et al., 2013). As reported by several recent studies, wrack piles might be significant source of greenhouse gas emissions (GHG) (Coupland et al., 2007; Lavery et al., 2013; Macreadie et al., 2017). Coupland et al. (2007) reported that, if left on the beach, seagrass wrack has an approximate emission rate of 6 μmol CO_2 m^{-2} s^{-1}. Moreover, Liu et al. (2019) shown that environmental conditions strongly influence GHG emissions by seagrass litter during the decomposition process: in presence of wet environment carbon rate increases compared to dry wrack. Indeed, moisture can enhance the loss of soluble materials via leaching and provide more suitable conditions for microbial community (Chen et al., 2000; Dick and Osunkoya, 2000; Nicastro et al., 2012). Moreover, seagrass leaves and roots can assimilate heavy metals present in environment due to high levels of anthropogenic pressure (Villares et al., 2016).

For these reasons, local council and coastal resource managers remove seagrass wracks often using heavy vehicles (such as machinery) which significantly alter the shoreline, influencing the beach morphology (Defeo et al., 2009; Vacchi et al., 2017; Figure 3). Moreover, the costs attributed to cleaning operations are very large and represent a huge expense for the income deriving from recreational and creative beach activities (Krelling et al., 2017).

Actually, in beaches all over the world, four different types of beached seagrass wracks management are applied according to the shore use:

1. maintenance of seagrass wracks on the beach. This management is applied on beaches that are not involved by an intense touristic flow, when seagrass organic matter is in very limited quantities or in shores with high naturalistic value (e.g.:

A and B areas of Marine Protected Areas, SPAMI areas or National Parks) (De Falco et al., 2008; Botero et al., 2015);

2. seagrass residues are removed in temporary stockpiles deposed behind dunes or in unauthorized/authorized plants. This management is applied also on least accessible touristic beaches or in shores little frequented by tourists. (De Falco et al., 2008). Moreover, this management is applied on beached exposed to erosion (ISPRA, 2010).
3. seagrass residues are collected from the beach or offshore, using dedicated equipment and nets, and are sink offshore. This management is not widely used because it involves noteworthy economic costs and the sunken residues can beach along the shoreline in the presence of intense and prolonged storms and winds (Bovina, 2009);
4. seagrass residues are collected and disposed in landfills. This is the technique most widely used in the management of beaches affected by an intense tourist flow (Zielinski et al., 2019). As mentioned earlier, heavy vehicles are used for cleaning operations, inducing significant alterations in morpho-dynamic beach profile (De Falco et al., 2008; Figure 3). Moreover, cleaning process removes not only beached seagrass detritus, but also propagules of plants and invertebrate species and a great amount of sand (Defeo et al., 2009). Seagrass residues collection, transport and disposal in landfill implicate that this management technique is among the most expensive (Mouat et al., 2010; Krelling et al., 2017). According to the Italian legislation, beached seagrass residues must be removed and treated as urban waste after merchandise analysis on collected material (Circ. 17/03/06).

To sum up, this type of beach management in particular present a lack of sustainability because:

- presents great economic costs,
- increase of GHGs due to seagrass wrack decomposition,
- use of non-renewable resources as petrol to transport the litter to landfills,
- involve a carbon loss from beach ecosystems.

Therefore, considering previous points, emerges a lack of synergy between marine and terrestrial environments and management beach system.

In several beaches, management systems applied beach certification schemes (BCS) in cleaning practices, which are based on the constant improvement in the performance, that evaluates the characteristics of a particular beach by using measurable compliance criteria (Botero et al., 2015). However, criteria used in BCS for cleaning practices paid lack of attention towards the ecosystem impacts of beach grooming, proving to be criticisable and inappropriate (Boevers, 2008).

Figure 3: Beach cleaning operations, Grado (NE Italy, North Adriatic Sea).
Picture: G. Misson

1.1.8. Seagrass litter reuse

Recently several studies explored the potential seagrass wracks reuse for the purpose of providing an "environmentally-friendly" application for beach management and reduce disposal costs. Below some of the applications suggested in several scientific papers are reported:

- composting. Indeed, the compost can be used as substrate in horticultural and floristic cultivation without using soil or as fertilizer. According to the experiences carried out in this field, this biomass composition integrates with other types of organic matter, allowing to obtain a compost that is appreciable for agronomic characteristics and rich in trace elements. This practice involves two critical aspects: the separation of sand from plant residues and salt removal. In Life project LIFE09 ENV/IT/000061, Cocozza et al. (2011) composted *Posidonia oceanica* leaves obtaining a compost with a good concentration of plant nutrients (e.g., N, Ca, K, Mg, Fe) and a heavy metal concentration well below Italian legislation limits. Moreover, superficial and internal salt presents in the residues was removed when it was necessary to restore the moisture of the piles (Cocozza et al., 2011). In Italy, the emanation of Dec. leg. 22/01/2009 "*Aggiornamento degli allegati al decreto legislativo 29/04/06, n. 217, concernente la revisione della disciplina in materia di fertilizzanti*" allowed to produce high quality compost from beached seagrass residues in a quantity not exceeding 20% of the initial mixture. The Polytechnic University of Valencia has produced a compost with excellent qualities using beached seagrass residues and vegetable waste and built a composting plant capable of treating about 15,000 m³/y of plant residues (Orquín et al., 2001);

- stabilize the dune system. This use of seagrass residues favours the colonisation of pioneer species on dunes and, subsequently, a positive feedback on sand accumulation and deposition. This technique involves the transportation of beached residues to coastal areas affected by erosion, in order to recreate dune morphology. The vegetable biomass must be separated from the anthropic material (plastics, glass, aluminium, etc.) by manual means. In Italy, INTERREG Beachmed project PosiDuNE, applied an innovative technique for coastal dunes restoration and protection using marine litter *Posidonia oceanica* (Cappucci, 2018);
- production of cosmetics and medicines. Recent studies have shown that seagrass extracts have antidiabetic, antioxidant and vasoprotective effects (e. g.: Newmaster et al., 2011; Rengasamy et al., 2013; Alamgir, 2018) For example, Nopi et al. (2018) demonstrated that *Thalassia hemprichii* contains polyphenolic compounds such as phenolic acids and flavonoids, known for their antioxidant properties. Moreover, seagrasses produce antimicrobial compounds that may act to reduce or control the microbial growth and there are many reports describing antibacterial, antiviral, anti-inflammatory, antidiabetic bioactive compounds isolated from seagrasses (e.g. Rowley et al., 2002; Hua et al., 2006; Ragupathi et al., 2010). Furthermore, seagrass residues are used for alginate and agar production (Kirkman and Kendrick, 1997);
- production of acoustic/noise isolation panels for buildings. Indeed, seagrass litter has a thermal conductivity similar to glass wool, a commonly used to insulate product. Vegetable biomass, before being used, must be processed (removal of salts, dry and thicken) in order to avoid corrosion or damage to

buildings. This application was experimented in project INTERREG IIIC 2000-2006 CosCo (Coastal Co-operation with sea grass and algae focus) in Germany, France, Poland and Denmark (EU, 2006);

- realization of design objects, such as vases, baskets, wickers, carpets. In this process the seagrass leaves are washed and intertwined (Font-Quer, 1990);
- manufacturing of substrates for textile/film/foam lamination. These processes have been studied by the Universities of Alicante and Andalucía in the project LIFE11 ENV/ES/000600 "SEA-MATTER - Revalorization of coastal algae wastes in textile nonwoven industry with applications in building noise isolation" (EC, 2015);
- production of nonwoven composites using wastes from *Posidonia oceanica* and other marine biomass as a raw material. These techniques were investigated by the University of Valencia in a LIFE project LIFE11 ENV/ES/000600. In this process, based on a paper-making technique, a dilute slurry of water and fibres from the marine vegetative waste is deposited on a moving wire screen and drained to form a web. The web is further dewatered, consolidated by pressing between rollers, and dried. This wet-laid reinforcement structure is processed, together with different renewable, non-toxic polymeric matrices, by means of a compression moulding system, to obtain the final composite products. The project confirmed the technical feasibility of the wet-laid process for producing nonwoven textile materials from marine biomass (EC, 2015);
- realization of biodegradable thermoplastic polymers. New developments include packaging applications for dry goods, pastes and fluids (UNEP, 2018);

- production of bio-absorbents (e.g.: activated carbons) for industrial processing waste, in particular to remove aromatic organic pollutants (β-naphthol and p-nitrophenol) which are toxic agents in wastewater. this process involves cellulose extraction and transformation into cellulose triacetate (Ncibi et al., 2009);
- realization of high-quality paper with seagrass leaves and *Posidonia oceanica* balls. *P. oceanica* is treated in order to extract cellulose and convert it into carboxymethyl cellulose. Moreover, it is necessary to integrate the pasta coming from the processing of the plant fibres stranded with other cellulosic material, in order to satisfy the physical and mechanical characteristics of the final product (Khiari et al., 2010);
- production of supplements in mixtures for animal food. Since ancient times beached residues of marine seagrasses were used as food supplements for farm animals, especially horses (Boudouresque and Meinesz, 1982);
- biochar production. Macredie et al. (2017) studied the potential conversion of seagrass wrack into biochar: this process exploits a thermochemical conversion of biomass in an oxygen-limited environment to create a solid material with high carbon content (Sohi et al., 2010). Preliminary results shown that *Posidonia australis* biochar had equal or higher carbon composition than other aquatic biochar products (Macreadie et al., 2017); moreover, biochar presents sever important nutrient (N, P, S, K, Ca) indispensable for plants. Chiodo et al. (2016) analysed the pyrolysis process in order to produce biochar: from the study it emerged that biochar yields produced by *P. oceanica* resulted comparable with those obtained by woody biomass, actually used for biochar production as fertilizer. Moreover,

biochar produced by seagrass resulted usable for soil application due to its characteristics of stability and alkalinity (Chiodo et al., 2016);

- gasification of *P. oceanica* residues to produce syngas for electricity production. Conesa and Domene (2015) studied this process considering different conditions (e.g., different relative humidity values, presence of saturated oxygen): the results showed a possible use of this residue in energy production, however it emerged the need to carry out more studies in order to optimize the process. Moreover, Deniz et al. (2015) demonstrated that the formation of gaseous products, gasification efficiency and yield distribution of produced gases from *P. oceanica* residues were intensively affected by biomass loading and temperature;
- biomethane production using anaerobic digestion. Several studies have estimated a bioenergy potential of some seagrass species (e.g.: Marquez et al., 2013; Ashraf et al., 2017). However, it emerged that it is necessary to continue research in this sector in order to optimize the biogas production process: actually, all experiments were carried out on a laboratory scale and not on an industrial scale. Moreover, the effect of salinity on anaerobic degradation is not clear: a high salt content of sea wrack biomass could inhibit anaerobic digestion using conventional terrestrial activated sludge as microbial seeds (Chen et al., 2003), however some results are conflicting. Such application not only improves the economic cost of waste management but is also beneficial by mitigating the harmful effects of anthropogenic methane production in landfills (Ashraf et al., 2017). As shown by Balata and Tola (2018), among the energy application of seagrass residues (incineration, pyrolysis/gasification and anaerobic digestion), the last is

considered the best solution for the population both in terms of economic and environmental sustainability.

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2. Thesis aims and outline

The thesis analyses the ecological decomposition dynamics of beached seagrass wracks, with focus on CO_2 and CH_4 emissions, and suggests a possible recovery of this litter for energy production through anaerobic digestion process. The study area was a beach of Grado municipality (Nord-East of Italy, in Adriatic Sea), where annually hundreds of tons of seagrass residues were collected during the Summer season. Indeed, in front of the shoreline, a seagrass meadow of *Cymodocea nodosa*, *Nanozostera noltii* and *Zostera marina* lies in good health.

The study has tree aims:

1. analyse the seagrass wrack decomposition dynamics in both controlled conditions and experimental field and determine CO_2 emissions as a function of temperature, salinity and water supply. This preliminary analysis has allowed to understand degradation process dynamics in order to select the best applicable technology for energy recovery of beached seagrass residues;
2. evaluate the use of seagrasses residues in anaerobic digestion using laboratory-scale digesters. A detailed physicochemical characterization was performed, focusing on elemental and proximate analysis. Different operating conditions were tested in the subsequent Biochemical methane potential (BMP) tests (in terms of Inoculum/Substratum ratio and use of wet or dry material) to evaluate the influence of these parameters on methane yield. Moreover, basic economical considerations were exposed and the possibility of using beach-cast residue after anaerobic digestion as soil fertilizer was analysed;
3. study temperature and salinity influence on methane emissions in anaerobic digestion of seagrass residues. Anaerobic tests were conducted reproducing typical environmental conditions observed in the studied area during the

Summer season. Moreover, CH_4 emissions in the environment during cold months were estimated considering the obtained laboratory results. Additionally, the changes in the relative microbial community abundance at the phylum and family level were investigated at different salinity concentrations, using metatranscriptomics sequencing, to evaluate the effect of this parameter on the specific community composition and consequently on CH_4 generation.

Part of the material contained in this thesis is taken from the following papers, realized during the PhD:

- Gloria Misson, Matia Mainardis, Fabio Marroni, Daniele Goi, Alessandro Peressotti. Environmental methane emissions from seagrass wrack and evaluation of salinity effect on microbial community composition using biochemical methane potential assays. Paper sent to Journal of Cleaner Production. Current state: submissions being processed;
- Gloria Misson, Matia Mainardis, Guido Incerti, Daniele Goi, Alessandro Peressotti. Preliminary evaluation of potential methane production from anaerobic digestion of beach-cast seagrass wrack: the case study of high-Adriatic coast. Paper sent to Journal of Cleaner Production. Current state: revisions being processed;
- Gloria Misson, Guido Incerti, Giorgio Alberti, Gemini Delle Vedove, Tiziana Pirelli, Alessandro Peressotti, 2017. Assessing the contribution of beach-cast seagrass wrack to global GHGs emissions: experimental models, problems and perspectives Geophysical Research Abstracts. 19: EGU2017-19416-1, 2017.

3. Effects of seagrass wrack litter decomposition dynamics on CO_2 emissions in both controlled conditions and experimental fields.

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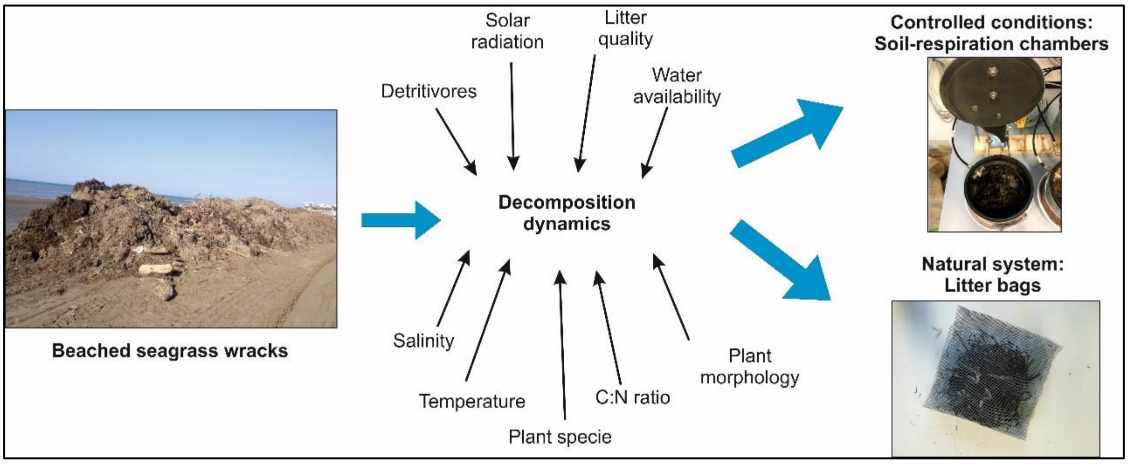
Manuscript in preparation

3.1. Abstract

Comparison of decomposition dynamics of *Cymodocea nodosa*, *Zostera marina* and *Nanozostera noltii* seagrass leaves have been performed in different experimental conditions. In laboratory decomposition was estimated using an automatic system of respiration chambers, whereas in field it was assessed using litter bags placed along the shoreline. Controlled setting allowed to explain the relationship between organic carbon loss and environmental factors, such as temperature, water availability and salinity. Moreover, it was shown that decomposition process was divided in three phases: loss of labile C, loss of stable compounds and degradation of recalcitrant substances. Seagrasses litter represented about 40% of total dry matter of wrack material, with a variable trend depending on seasonality. The deposition along the shoreline of seagrass residues was maximum during the Winter period, with an estimated biomass of about 12 kg m^{-2}. Carbon organic loss was higher in field than experimental conditions, due

the presence of other physical and biological factors that influenced decomposition process.

3.2. Graphical abstract



3.3. Keywords

Seagrass meadows, beached seagrass wrack, carbon cycle, litter bags, respiration chambers.

3.4. Introduction

Seagrass meadows are among the most important ecosystems in marine habitat due to their capacity of storing high amounts of carbon, also for millennia, and their important role in many coastal processes (Simeone, 2008; Mcleod et al., 2011, Vassallo et al., 2013; Ruiz-Frau et al., 2017). Like many terrestrial higher plants, marine seagrasses lose their old leaves during annual or inter-annual senescence, and a significant proportion of these residues is transported in surface waters and washed up on shores by surf, tides and winds (Mateo et al., 2003; Mateo, 2010). Beach-cast seagrass wracks present several ecosystem services: reducing wave impact, protecting beaches from erosion, providing habitat to birds and invertebrate species that colonize the shorelines, and being a primary food resource for beach detritivores (Defeo and McLachlan, 2005; McLachlan and Brown, 2006; Vassallo et al., 2017; Vacchi et al., 2017). The amount of beached seagrass litter deposited on shores varies considerably depending on several factors such as healthy state of the meadow, seagrass species, sea weather conditions, climate and seasonality (Mateo et al., 2003). The quantity of seagrass residues exported on beaches ranges between 10 and 55% of the total meadow primary production (Boudouresque et al., 2012); however, exported biomass can reach 95% of annual leaf production (Ochieng and Erftemaijer, 1999). accumulation of seagrass wrack on beaches, following meadows degradation, can negatively impact tourism (Kienzlman et al., 2003; Davenport and Davenport, 2006). Therefore, wrack piles are frequently collected and disposed of in landfills or biomass waste facilities, and the adoption of these management practices implies substantial environmental and economic costs (Defeo et al., 2009; Zielinski et al., 2019). Recent studies highlighted that seagrass wrack piles might be a significant source of greenhouse gases emissions (GHGs), with global consequences on carbon cycle due to increase in atmospheric CO_2 and CH_4 concentrations (Coupland et al., 2007; Lavery et al., 2013; Macreadie et al., 2017; IPPC, 2019). Even though quantitative estimates of both seagrass coastal distribution and residues disposal to seashores are partially available, at least at regional level, the assessment of their contribution to global GHGs emissions is still lacking (particularly regarding CO_2 flow), due to a knowledge gap about the effects of peculiar environmental conditions of beach ecosystems on seagrass decay rates.

Plant litter decomposition plays an important role in C cycle regulation and in its transfer to the various biosphere, hydrosphere, atmosphere and lithosphere compartments (Houghton, 2007). The dynamics of this linkage are regulated by decomposer organisms: their decomposition efficiency depends both on the physico-chemical characteristics of organic matter and on the climatic and environmental conditions which can slow down or speed up the degradation process (Don and Kalbitz, 2005; Campbell et al., 2016). Indeed, litter quality is fundamental in determining the decomposition rate (Meentemeyer, 1978). Soluble substances and labile compounds are fast degraded due to the need of remarkable quantities of carbon and nitrogen, essential for bacteria growth (Incerti et al., 2011). Indeed, the C/N ratio greatly influences the initial decomposition rate. Carbohydrates, proteins, a fraction of microbial biomass, water-soluble substances and other simple compounds are first decomposed, thus modify inorganic matter characteristics (Berg et al., 1987). Subsequently, bacteria decompose most recalcitrant and resistant substances such as lignin, cellulose and holocellulose (Rutignano et al., 1996). In this phase, as reported by Berg et al. (1982), the nitrogen litter concentration is critical for lignin degradation: the lignin decomposition rate is lowest for N-rich litters and highest for N-poor ones. In the last stage the most stable compounds are decomposed including suberins, resins and waxes, which are slowly degraded (Rutignano et al., 1996). As reported by several studies, climatic and environmental context is critical in decomposition process: temperature, oxygen availability and water availability can strongly influence microbial activity (Berg, 2000; Fourqurean and Schrlau, 2003; Dang et al., 2009; Bradfort et al., 2016). Indeed, high temperature and moisture scarcity can firmly limit bacteria growth (Fioretto et al., 2005), because they determine the susceptibility of litter compounds to microbial attack (Rovira and Vallejo, 2007). Furthermore, in transitional environments such as beaches, the decomposition process is influenced by salty water: high salt concentrations can significantly slow down the decomposition process. (Mendelssohn et al., 1999; Quintino et al., 2009). Litter decomposition has been widely studied in terrestrial and freshwater environments, however, studies of leaf litter decomposition in transitional ecosystems, such as beaches, are less common (Mateo and Romero, 1996; Bayo et al., 2005; Sangiorgio et al., 2008).

In this study, the carbon loss decomposition process of beached seagrass litter was investigated in relation to different environmental factors, such as temperature, water supply and salinity. The experiments were conducted both in laboratory and in field in order to distinguish the effective interaction of different environmental agents on litter decomposition and compare the estimated results with real carbon loss obtained in the field. Moreover, the deposition dynamics of beached seagrass residues were also investigated in relation to seasonality and chemical characteristics of plant material beached on the shoreline.

3.5. Materials and methods

3.5.1. Study area

Beached seagrass was collected along the coastline of Friuli Venezia Giulia, an administrative region of NE Italy (Grado, Adriatic Sea, NW Mediterranean; Figure 4). The seagrass meadow in Grado is composed by different patches of *Cymodocea nodosa*, *Zostera noltii* and *Zostera marina* settled on a sandy bottom (Fallace et al., 2009). The upper limit is close to the coastline and the meadow presents a high density between bathymetries of 3 and 4 m depth, then it shows a medium coverage to the bathymetry of 6/7m (Fallace et al., 2009). The meadow is interested by anthropogenic impacts due to coastal development and tourism, that is mostly relevant during the Summer period. The seafloor of the study area presents a shallow depth with a low bathymetric gradient, furthermore the sediment is dominated by bare sand. The marine water has a high temperature gradient with a minimum of 5°C in January and a maximum of 27°C during the summer, with salinity values ranging from 28 to 34 ‰ (ARPA Friuli Venezia Giulia meteo, 2018). Considering the hydro-dynamism, a cyclonic circulation from South-East to North-West is present near the coastline (Artegiani et al., 1997).

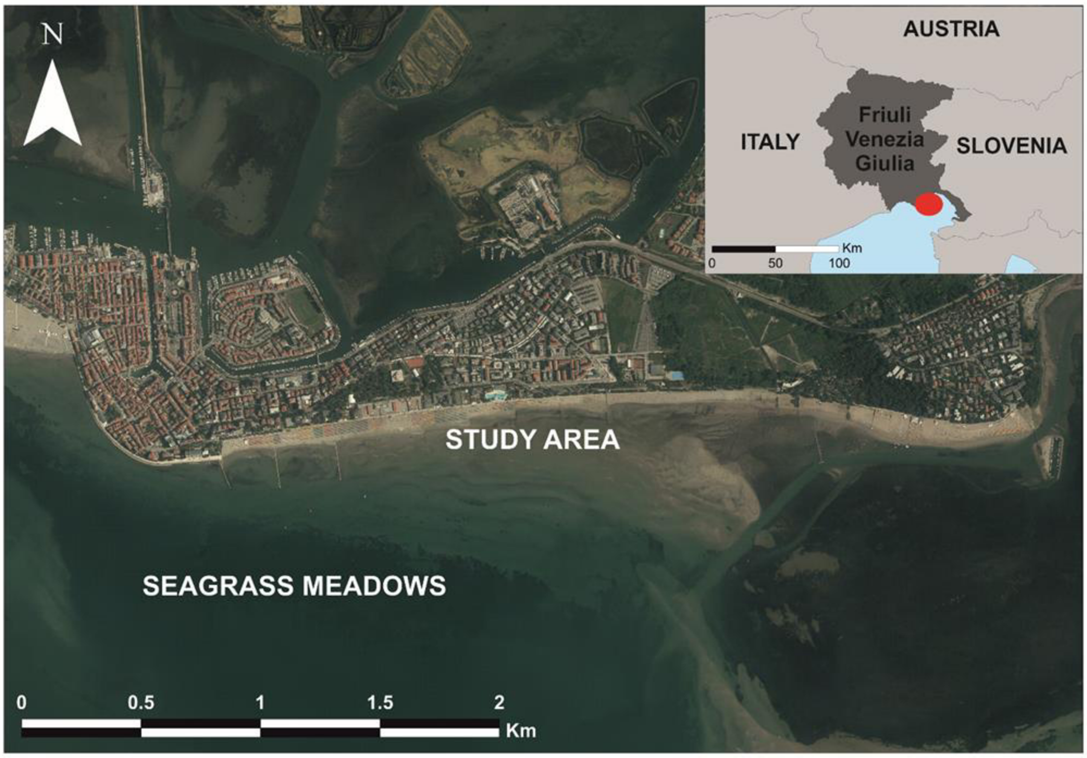


Figure 4: Overview of the study area (Northern Adriatic Sea), Friuli-Venezia Giulia geoportal.

3.5.2. Sampling

Wrack samples were collected along 8 transects by random point sampling once a week. The field work was carried out from Spring 2016 to Spring 2017. The collected material was washed taking care not to disperse the sand present in the samples. Subsequently samples were divided into different categories: sand, wood, seagrasses, algae, other (e.g. shells, plastic, stones). Seagrasses were isolated from the rest; each wrack sample and separately shoots and rhizomes were analysed, after taxonomic identification. Samples of seagrass green litter were cleaned by epiphytes and washed with deionized water. All wrack categories were weighted, dried at 30°C until constant humidity and weighted again in order to determine the relative humidity.

3.5.3. Litter decomposition experiment in controlled condition

CO_2 fluxes can be measured by different techniques, that influence the apparent rates of techniques (Livingston and Hutchinson, 1995). A system that highlighted the high spatial and temporal heterogeneity in rates of CO_2 efflux was used: the Uniud-SR is an automated closed dynamic system that provides high temporal resolution measurements of CO_2 efflux over long period of time (Delle Vedove et al., 2007; Figure 5). Efflux was automatically and continuously measured for 5 minutes every 2 hours using open-top chamber system operating with 12 chambers in parallel. Each chamber consisted of a steal collar (20 cm of diameter and 8 cm height) and an DC motor closing a steal lid. In the steal collar of each respiration chamber, a layer 1 cm thick of clean sand (about 140g) was placed, overlaid by seagrass dry leaves litter (20 g dry weight) partially covered with 70 g of sand: sand and seagrasses residues quantity reproduced natural conditions of the sampling site. To remove the sand from the organic matter, the inorganic material was cleaned with dust remover. 6 replicates were treated with fresh water (FW) and 6 with marine saltwater (SW), periodically collected at the same sampling site. The treatment with fresh and salty water reproduced the natural cycle of wrack wetting by the waves (SW) and rains (FW), that washed out the salt by beached material. In order to maintain an optimal moisture level for microbial respiration activity, each decomposing litter replicate was daily watered at field capacity for 165 days with either FW or SW, according to the initial treatment. Each replicate was incubated at room temperature conditions, in presence of a thermocouple sensor to record actual temperature. Moreover, the laboratory was obscured by direct rays of the sun, in order to minimize its influence on temperature increase and on decomposition process speed.

Figure 5: Uniud-Soil Respiration system

3.5.4. Field experiment of litter decomposition

Decay experiment was determined by measuring litter mass loss and was performed using litter bags methods (Fioretto et al., 2005). Mesh bags (1 mm mesh, 20 x 20 cm²) contained 10 g of dry weight (DW) of seagrass leaves green litter and were placed along 8 transects and random points. Transects were positioned perpendicularly to the shoreline, while in the random points the litter bags were placed both on the sand and inside wrack piles. Three mesh bags were used for each combination of time and position and they were collected after 15, 30, 60, 90, 120 days of incubation. The remaining material was dried at 70°C, removed from sand and separately weighted. The material in each litter bag was divided in tree subsamples and it was prepared for elemental analysis.

3.5.5. Laboratory and statistical analysis

Seagrass leaves litter was finely ground in order to analyse carbon and nitrogen percentage at the beginning and at the end of the experiment. The total carbon (C) and total nitrogen (N) contents were determined by elemental analysis (Vario Micro Cube, Elementar). Living and detrital leaf samples collected along shoreline were processed in the same way as beach-cast detritus.

One-way ANOVA was used to analyse data variability among respiration chambers and litter bags. Where ANOVA results where significant at $p = 0.05$, differences among mean values were determined using Tukey's honesty significant difference test. Beached seagrass data were submitted and analysed using the software STATISTICA 7 (StatSoft Inc., Tulsa, OK, USA) in order to obtain the deposition seagrass litter model.

3.6. Results and discussion

3.6.1. Characterisation of beached material and deposition dynamics of seagrasses residues

The composition of beached material changed according to seasonality, as reported in Figure 6A. Wracks presented an annual percentage of about 50% dry matter (DM) of sand, with a maximum during the Summer period (58.4 ± 14.0% DM) and a minimum in Winter (40.2 ± 6.1% DM). Seagrasses represented about 40% DM of wrack material with a minimum in Spring (32.2 ± 6.9% DM) and a maximum during Winter (44.2 ± 10.0% DM). Seagrass samples showed a relative higher humidity in Summer (58.8 ± 17.72) and lower in Winter (47.2 ± 19.8), while in Spring and in Autumn it presented intermediate values (55.81 ± 15.5 and 49.5 ± 21.6, respectively).

Wood, algae and other material shown a minimum percentage in wracks: algae presented a pick in Spring (6.7 ± 1.36% DM), whereas wood and other material exhibited a maximum in Winter (7.9 ± 4.8% DM; 3.1 ± 2.1% DM, respectively). Analysing seagrasses residues in detail, they consisted of 30.6 ± 10.5% DM of leaves and 2.4 ± 1.2% DM of rhizomes in Summer (on a seasonal total of 32.6% DM), while in Winter leaves represented about 30.9 ± 13.2% DM and rhizomes 13.2 ± 3.1% DM (on a seasonal total of 44.2% DM; Figure 6B).

Considering the average values of all seagrasses samples, *Cymodocea nodosa* constituted 51.2 ± 21.4% of seagrass organic matter (composed by 76.5% leaves and 23.5% rhizomes). *Zostera marina* and *Nanozostera noltii* were found in lower percentage, 28.6 ± 16.4% (leaves: 82.9%, rhizomes: 17.1%) and 20.2 ± 12.6% (leaves: 62.3%, rhizomes: 37.7%), respectively.

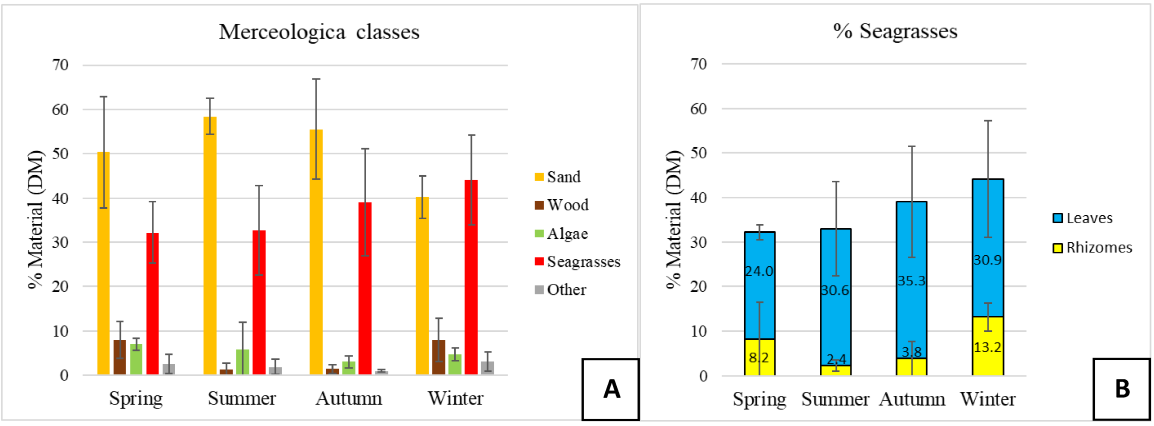


Figure 6: Merceologica classes of beached material (A) and seagrass percentage in wracks (B). DM: dry matter.

The significant effect of seasonality on deposited material composition is influenced by weather conditions (ARPA Friuli Venezia Giulia meteo, 2018). Indeed, in Winter there are intense storms that tear a large number of rhizomes and shred leaves beach themselves on the shoreline. Instead, in Summer beached leaves increased due to maximum vegetative period of seagrass meadows and to the growth of plant marine biomass. Indeed, as reported in Figure 7, considering the period between April 2016 and April 2017, beached seagrasses presented a pick in Summer, with a calculated seagrass biomass equal to 11.8 kg m^{-2}, significantly higher than Winter season with a range between 2-8 kg m^{-2} ($p < 0.05$).

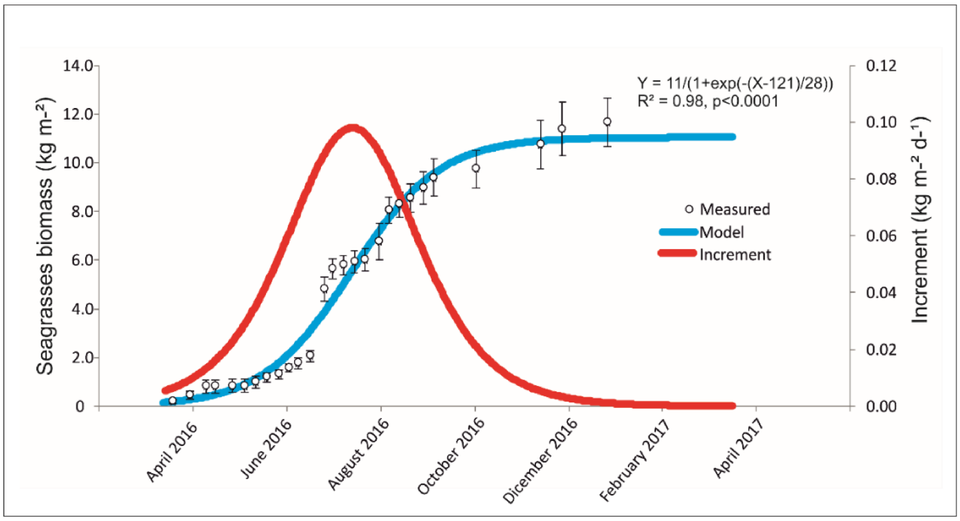


Figure 7: Deposition dynamics of seagrass biomass in study area during the period April 2016-April 2017.

Considering the different sectors in which the study area was divided by the presence of breakwater barriers, the average dry values of beach cast detritus were lower in the sectors close to "Lungomare Nazaro Sauro" and maximum along the shoreline close to "Banco Mula di Muggia": during the period April 2016-April 2017, in the last three transects it was collected on average the 53±21% of beached material. The differences between spatial scale (particularly between the first and last transects) reflected the large differences in accumulation patterns between areas. These patterns differences were caused by waves direction and the prevailing winds of Libeccio (South-West), which transported the material towards "Banco della Mula di Muggia" (Baucon and Felletti, 2016). The present results confirmed the important role play by winds, tides, hydrodynamics and morphological characteristics of the beach in governing detritus transport and export (Mateo et al., 2003; Mateo, 2010).

3.6.2. Beached seagrass litter decomposition in controlled conditions

Obtained data were reported in Figure 8. There were large differences between the decay rates of samples treated with freshwater and marine water (p =0.001). Both treatments shown an exponential decreasing decomposition trend, however, samples treated with freshwater presented a greater CO_2 emission (Figure 8A and 8B). As reported by several studies (e.g. Ochieng and Erftmeijer, 1999; Fourqurean and Schrlau, 2003; Incerti et al., 2011), the initial rapid biomass decay rate was caused by the degradation of the most labile fraction of organic matter by microbial community. However, the decomposition process was significantly affected by environmental factors such as temperature, water availability and salinity.

The temperature increased during the experimental campaign, due to the rise in the external seasonal temperature (Figure 8C). The average water content in samples decreased in correspondence to the temperature increase, which induced a greater evaporation (Figure 8D). However, daily reaching the field capacity, the moisture present in samples favoured organic matter degradation by microbial community.

Figure 9 shown the correlation between efflux (µmol CO^2 m^{-2} s^{-1}) and temperature (°C). Indeed, temperature is an important factor in controlling decomposition dynamics in presence of water availability. Analysing this relationship, it is possible to divide the decomposing process into three different phases: they represent the decomposition of labile substances (initial phase), recalcitrant compounds (intermediate phase) and stable substances (final phase). Moreover, Figure 9 underlined that in the first phase there was an inverse relationship between efflux and temperature. Indeed, when temperature increased, CO_2 emission decreased. This relation was present both in samples treated with freshwater and in samples treated with marine water. In the second and third phase of decomposition process, there was a positive correlation between efflux and temperature: as the temperature augmented, CO_2 emissions increased. However, this aspect was more evident in the samples treated with freshwater.

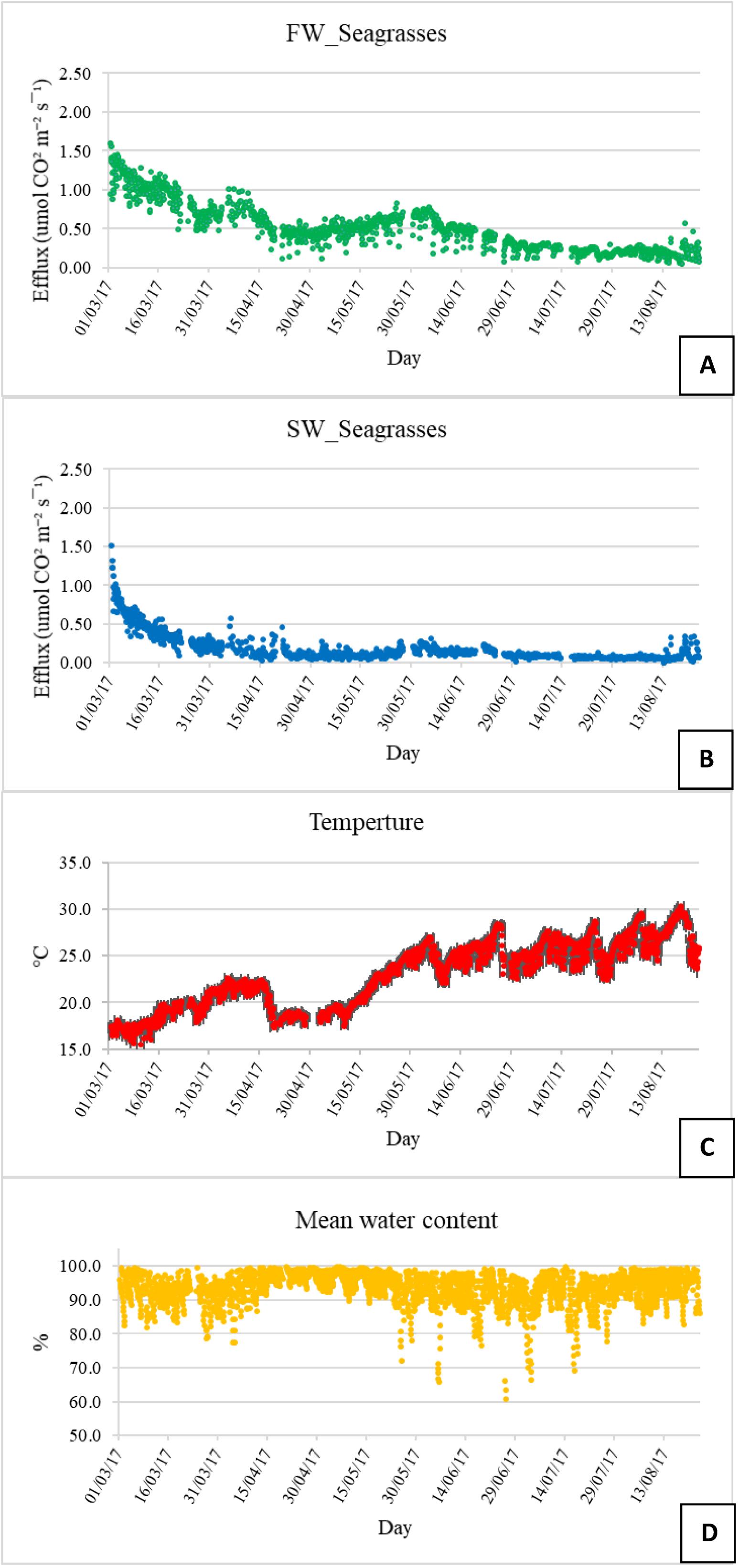


Figure 8: Efflux (Efflux (umol CO^2 m^{-2} s^{-1}) of samples treated with freshwater and marine water (A and B), mean values of temperature in °C (C) and the percentage of mean water content (D).

Indeed, during the second phase, samples treated with saltwater had a high salts concentration, which slowed down the mycobacterial activity.

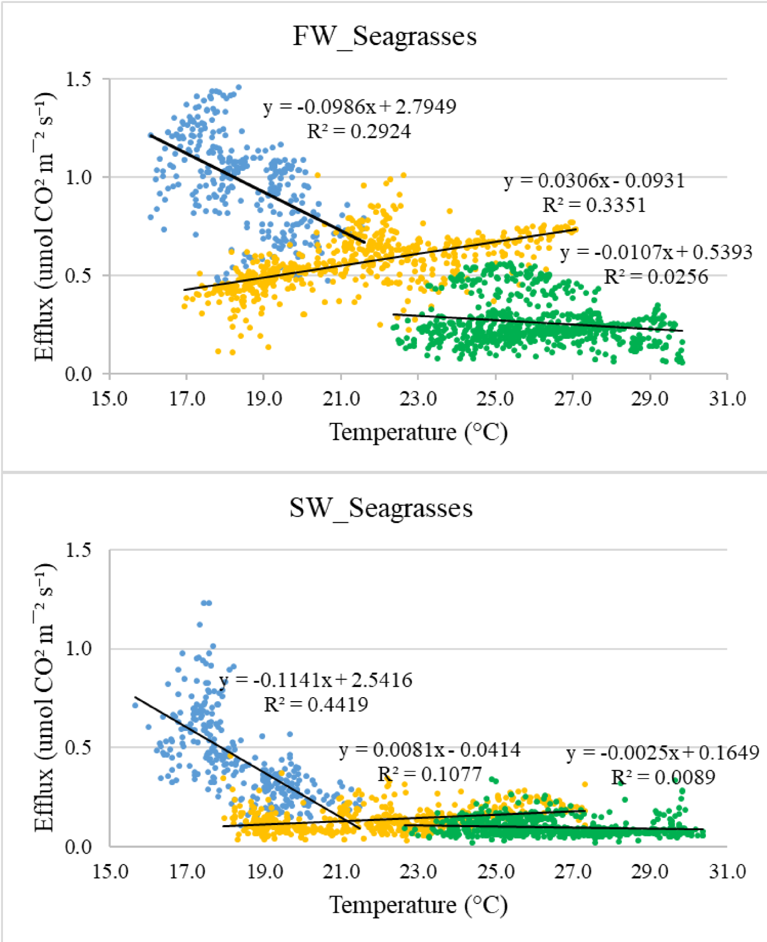


Figure 9: Correlation between efflux (umol CO^2 m^{-2} s^{-1}) and temperature (°C) in samples treated with freshwater and marine water.

The remaining biomass data were modelled as a negative exponential decay function. Moreover, it emerged that FW seagrass samples decomposed faster (%C loss d-1= 0.10) than either SW seagrass (%C loss d-1= 0.03). After 165 days of decomposition, FW seagrass samples lost about 16.9 ± 2% of total initial carbon present in biomass, compared to 6.2±1% of SW seagrass samples (Figure 10). The slowing down of decomposition process in samples treated with saltwater was caused by the increase in salinity concentration during the experiment. It was shown that a salt concentration higher than 48 ± 1 g/l slows down organic matter decomposition and a salinity concentration equal to 54 ± 2 g/l blocks this process. Therefore, it emerges that the salt concentration is a determining factor in organic matter decomposition in presence of optimal temperature conditions and water availability. As reported by Quintino et al. (2009), along increasing salinity gradient, the biomass decomposition diminished directly with salt concentration: the remaining dry weight biomass was higher when marine conditions prevailed and reached the lowest value in freshwater environment.

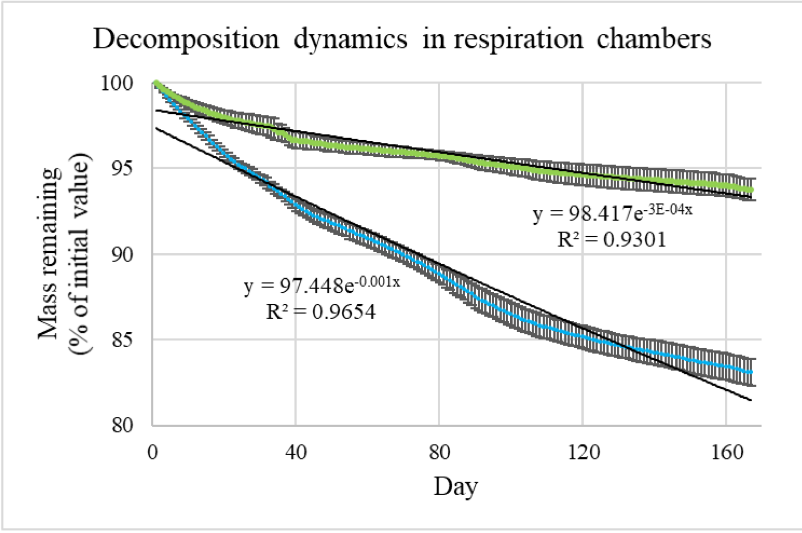


Figure 10: Decomposition dynamics in respiration chamber. The blue line represent %C loss in seagrass samples treated with freshwater and the green line reported %C loss in samples treated with marine water.

3.6.3. Beached seagrass litter decomposition in environmental conditions

Field decomposition data obtained by litter bags were reported in Figure 11. The degradation process was represented as a decreasing exponential function: after a period of 120 days, around 32% of the initial organic carbon (OC) was already degraded. The first part of the degradation process was characterized by a rapid loss of organic matter, lasting for about three months. The second step could be described as slower decomposition phase of more refractory material. The %C in litter bags decreased rapidly; in contrast, the N content increased very slowly with time from 2.0 ± 0.1 to 2.9 ± 0.2 of dry weigh. Correspondingly, C/N ratio of remaining seagrass litter decreased as reported in Figure 12.

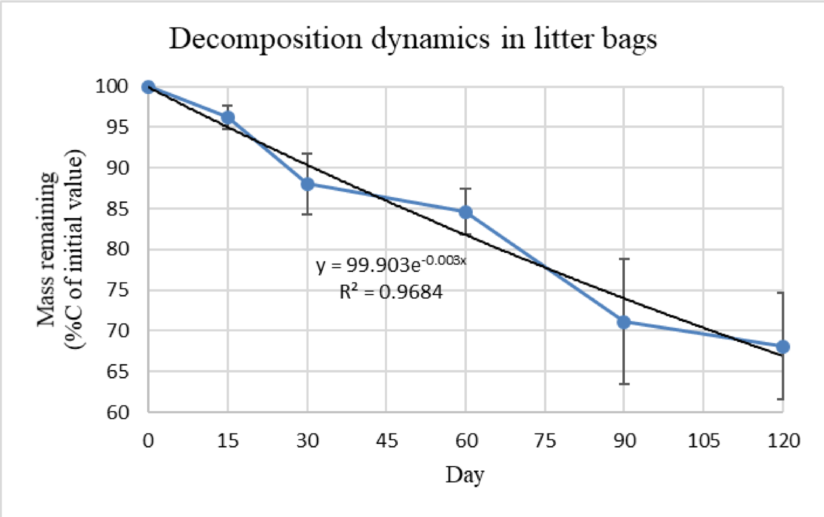


Figure 11: Decomposition dynamics in litter bags collocated in study area.

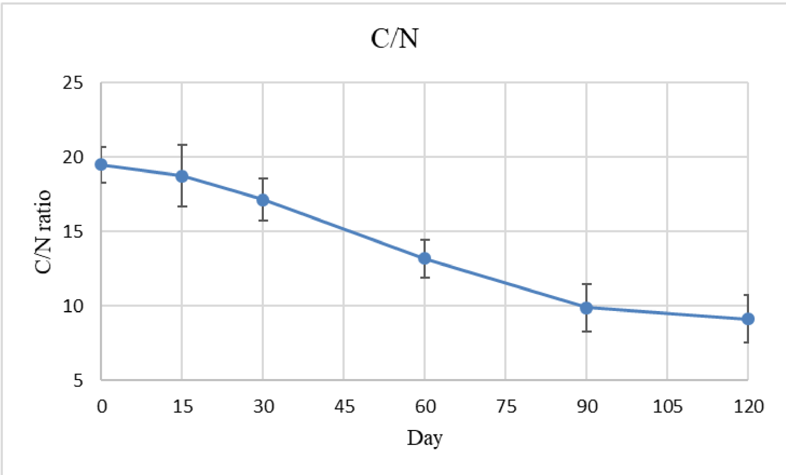


Figure 12: C/N ration in seagrass litter bags.

The presence of a higher nitrogen concentration in senescent leaves than fresh seagrass litter was found in other studies (e.g. Mateo and Romero, 1996; Fourqurean et al., 2003). Mateo and Romero (1996) reported that bacteria growth was faster in senescent leaves than beached seagrass litter, due to a potential N-limitation. Indeed, as reported in several studies, C/N ratio significant influenced the degradation process both in terrestrial and in marine environments (e.g. Berg et al., 1982; Manzoni et al., 2008; Yue et al., 2016). C/N ratio is one on the main intrinsic factors influencing decomposition, in addition to plant species, plant morphology, lignin content and tannic acids and polyphenols presence (Austin and Vivanco, 2006).

Figure 13 explained the relation between the biomass remaining in seagrass wracks in litter bags and the daily mean temperature in the same period and area. From the reported data emerges a remarkable correlation between two variables: as reported by several studies degradation is significant related to temperature (e.g. Burke et al., 2003; Davidson and Janssens, 2006; Frey et al., 2013; Bonanomi et al., 2014). Relating rains and seagrass biomass remain we do not obtain significative correlation ($p > 0.05$). This result can be explained because beached residue was wetting not only by rains but also by waves, especially in high tide. However, this data is not available and therefore it was not possible to investigate obtained results.

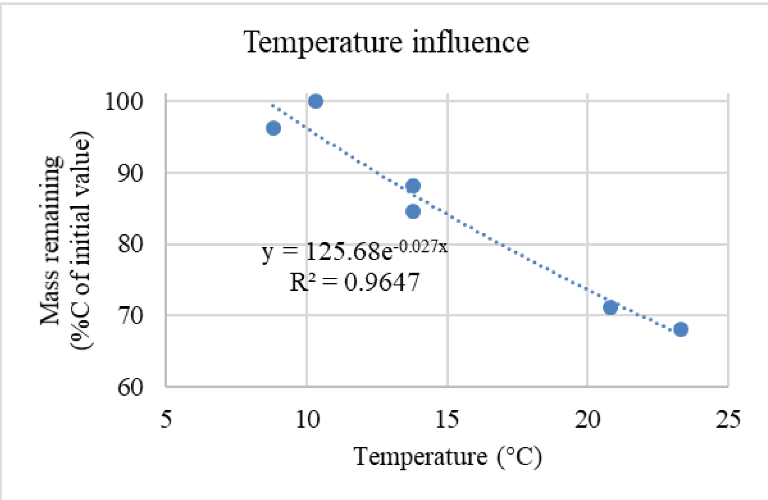


Figure 13: Relation between temperature and biomass remain in litter bags in field experiment.

Comparing data obtained in experiment conditions and in field in 120 days, emerges that along shoreline degradation process is faster, with a %C loss greater than 17% relate to controlled settings. This difference is related to the presence of other chemical-physical factors than increase the degradation process along shoreline. Those factors explain the differential response between field obtained data and laboratory measurements. Indeed, in field, the presence of meiofauna rises weight loss and accelerate decomposition of seagrass wracks (Mateo and Romero, 1996). As reported by Como et al. (2008), seagrass leaves detritus presents a rich macrofaunal assemblages due to structural complexity and facilitative role of seagrass favouring animal colonisation and increasing diversity of associated detritivores. Moreover, solar radiation impacts on the decomposition detrital leaves of seagrass. As shown by Vähätalo et al. (1998), brown seagrass leaves absorbed more than 90% of photons in the near ultraviolet and blue part of the spectrum, resulting more sensitive to photochemical reactions than fresh beached leaves. Furthermore, solar radiation induces some fragmentation of leaves, reducing structural barriers in leaves and exposing substratum to the colonization by bacteria. Therefore, such photo-reactions induce an increase of carbon loss from seagrass wracks (Vähätalo et al., 1998).

3.7. Conclusions

In this study were applied two techniques in order to evaluate the decomposition process of seagrass litter both in controlled environment and along shoreline. In laboratory seagrass leaves were treated with fresh and salt water in order to reproduce natural conditions and CO2 efflux was measured using automatic soil respiration chambers. Decomposition was higher in material treated with freshwater than those treated with salty water, due the increase of salt concentrations during the experiment. Indeed, obtained data shown that a salinity equal to 48 ± 1 g/l slowed down the seagrass leaves degradation and this process was stopped with concentrations around 54 ± 2 g/l. Experiment data underlined the effects of physical factors controlling decomposition, such as temperature, water availability and salinity concentration. Litter bags containing seagrass leaves residues were used in field experiments. Along shoreline the degradation of organic material was faster than experimental setting: in 120 day the remain biomass was lower of 17% than remain mass in soil respiration chambers. This increase was caused by the presence of other physical and biological factors that accelerate decomposition process, such as the presence of detritivore organisms and solar radiations.

This study has allowed to further understand the decomposition dynamics of beached seagrass residues in relation to several environmental factors that interact with this process. Future investigations in field conditions allow to explain other gaps of effects of environmental factors on decomposition in natural systems, for instance the variability of seagrass leaves degradation along spatial scale and the effects of biomass characteristics.

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4. Preliminary evaluation of potential methane production from anaerobic digestion of beach-cast seagrass wrack: the case study of high-Adriatic coast

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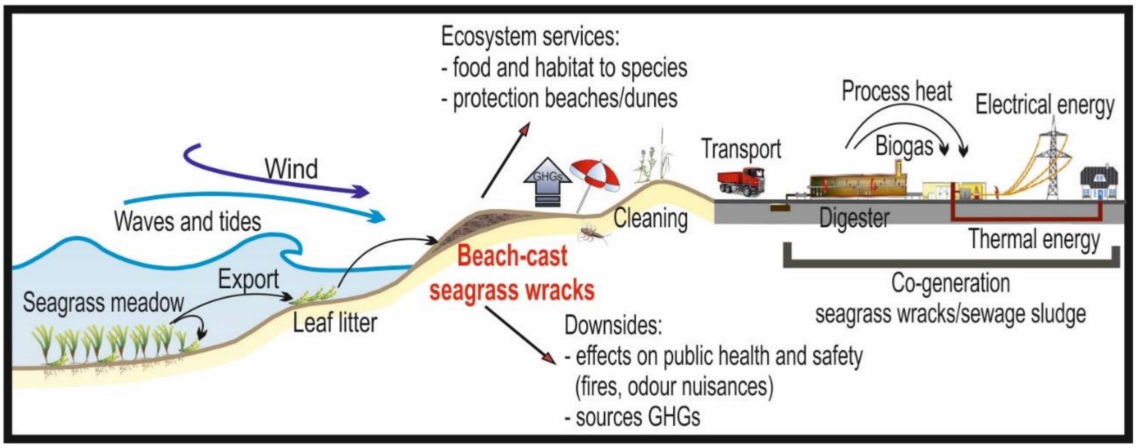
4.1. Abstract

Seagrass meadows are important productive ecosystems; during the Summer period in touristic beaches, such as those located in the high Adriatic coast, they are removed from the shoreline and disposed in landfill. This study investigated anaerobic digestion potential of beach-cast seagrass wrack, considering the physicochemical characteristics of the substrate and analysing heavy metal presence in digestate, with the aim of transporting the material to local wastewater treatment plant to increase biogas yield from excess sludge digestion. The methane production obtained from seagrass wrack was compared with three theoretical models. Anaerobic digestion assays proved that seagrass wrack had a good methane potential of 103.1-262.3 NmL CH_4/g Volatile Solids (VS), depending on substrate humidity and applied inoculum-to-substrate ratio.

Predictive models, based on elemental composition and proximate analysis, successfully estimated methane yields; moreover, heavy metal concentration in digestate was low, boosting for digestate agricultural reuse. A simplified energy analysis revealed that transport to local wastewater treatment plants and use in anaerobic digestion would provide up to 245,000 Nm^3/y of methane, with an estimated economic income of 33,500-193,300 €/y, considering seagrass production (1,465-8,454 t/y). Actual yearly costs sustained for landfill disposal was about 117,200-676,320 €/y. Seagrass use would compensate for the lack of excess sludge encountered during the cold season, allowing the digester to operate more continuously, increasing biogas production and reducing the energy need of the analysed plant.

4.2. Graphical abstract



4.3. Keywords

Beach-cast litter; Seagrass; Anaerobic digestion; Energy production; Biochemical methane production tests.

4.4. Introduction

Seagrass meadows are important productive coastal ecosystems: they are considered major carbon sinks, with an average annual seagrass production estimated as 1,012 g dry weight (DW)/m^2 y (Duarte and Chiscano, 1999). Marine seagrasses lose their old leaves during annual or inter-annual senescence and a significant fraction of these residues is transported in surface waters and washed up on shores by marine surf, tides and winds (Jiménez et al., 2017). Therefore, seagrass wrack piles can cover wide areas with a height ranging from few centimetres up to several meters (Mateo et al., 2003). The amount of produced necromass (i.e. the material lost during the senescence cycle) changes depending on local factors (i.e. hydrodynamism, depth and characteristics of seagrass meadows) and studied specie: this value has been estimated as 60-100% of the seagrass primary production, while the fraction exported on-shore ranges from 15% to 55% of annual leaf production (Cebrian and Duarte, 2001; Boudouresque et al., 2016). Seagrass wrack piles have important ecological functions (Mateo et al., 2003; Vacchi et al., 2016; Del Vecchio et al., 2017), however, in most cases seagrasses are removed in beaches of touristic interest. This is due to odour nuisances that develop from organic matter fermentation or the possibility of fires generation, with negative effects on public health and safety (Parente et al., 2013). Cleaning or grooming are common adopted practices to remove the residues, often done with heavy equipment (such as tractors), and the wrack material is disposed in landfills (in the absence of alternative arrangements) (Defeo et al., 2009). This modus operandi, currently applied also in the analysed high-Adriatic basin, does not involve a sustainable beach management because of the organic matter removal (especially carbon) from the beach ecosystem, with consequent carbon loss, coupled with high economic costs and sand withdrawal.

In the last decades, the interest in environmental protection was constantly increasing worldwide; European Union (EU) approved several directives, focusing on advanced biofuels use, with the goal of decreasing greenhouse gas (GHG) emissions in energy production and collection, treatment and recycling of municipal waste, in order to reduce pollution and decrease feedstock use (EC, 2017). Anaerobic digestion (AD) is one of the most relevant renewable energy production technologies. AD is a biological process in which organic matter is decomposed by a microbial consortium in an oxygen-free environment (Pain and Hepherd, 1985). AD is a process that occurs in many natural anoxic environments, but it can also be applied to produce energy in biogas and biomethane forms (Wall et al., 2017). Biogas is composed of 50-70% methane and 30-50% carbon dioxide and can be used locally to produce electricity, heat, or both in a combined-cycle process (Grando et al., 2017), and even upgraded to biomethane. Seagrass use for energy production is being considered throughout the world, where the presence of large amounts of beached seagrass is a problem for the tourism economy for aesthetic and safety reasons (Balata and Tola, 2018).

In the analysed area (Northern Adriatic coast, Italy), important touristic municipalities are present, characterized by high people presence (>100,000 inhabitants) during Summer, while effective resident population in the cold months is significantly lower (about 10,000 inhabitants): local wastewater treatment plants (WWTPs) need to face extreme flowrate and load variations. It was seen in plant operations that AD of excess sludge lacks substrate during the cold months and actual management approaches include discontinuous digester operations. A more sustainable approach should involve the use of complementary substrates to allow the digester to operate more smoothly and continuously. Given the fact that consistent amounts of beach-cast seagrass are available at low distance from the analysed digester, the possibility of storing and using this material in AD to boost the process was studied.

Biochemical methane potential (BMP) tests are the most widely used method to estimate the maximum methane production from a given substrate (Raposo et al., 2011), that is one of the key parameters to study the feasibility of full-scale AD application (Da Silva et al., 2018). BMP procedure involves adding small amounts of selected inoculum and substrate into serum bottles, creating anaerobic conditions and measuring gas production over time; a cumulative gas production curve is obtained and BMP value is typically expressed as function of added volatile solids (VS) (mL CH_4/g VS) (Pearse et al., 2018). Beside cumulative CH_4 production, the analysis of methane production kinetics is significant to analyse and scale-up AD process: in the first digestion days, the most easily degradable fraction is expected to be degraded (Mainardis et al., 2018), and methane production is consistent, while successively the conversion of slowly degradable molecules takes place. BMP tests require long times to get robust data; new methods were suggested to obtain reliable data on methane yield, such as near-red spectroscopy and aerobic respirometry (Ward, 2016), as well as statistical analysis of BMP data results (Stromberg et al., 2015), and were applied also in the present study.

The digestate from AD process is rich in N and P, that are essential soil nutrients (Stiles et al., 2018), and thus is often reused in agriculture; however, the presence of potentially toxic elements, such as lead (Pb), zinc (Zn) and copper (Cu), must be avoided (Coelho et al., 2018). In this work, heavy metal concentration was detected after BMP tests at different applied inoculum to substrate (I/S) ratios, to underline the effect of seagrass addition on heavy metal presence in the digestate and allow a safe agricultural application of the material.

This research was aimed at carrying out a preliminary campaign to assess the obtainable methane production in AD from beach-cast seagrasses, coming from the high Adriatic basin, to improve actual waste management, that actually includes landfill conferral, with huge disposal costs and environmental impact. Recent literature studies (Balata et al., 2018; De Sanctis et al., 2019) analysed energy potential of *Posidonia Oceanica* residues for AD application; the high reported lignin content in the material reduced AD process applicability (De Sanctis et al., 2019). However, the possibility to reuse this material in existing digesters to increase methane yields, reducing investment costs, was not considered. Moreover, seagrass composition in the High-Adriatic coast is extremely different that that reported in available literature studies. Finally, a complete heavy metal characterization on the digestate after AD was not performed in available literature.

The aim of the work was to evaluate the technical feasibility of using seagrasses in local WWTPs where anaerobic digesters suffer from a low substrate availability, increasing biogas yield. A detailed physicochemical characterization was performed, focusing on elemental and proximate analysis. Different operating conditions were tested in the subsequent BMP tests (in terms of I/S ratio and use of wet or dry material) to evaluate the influence of these parameters on methane yield. Simplified regression methods were applied to analyse the fitting between theoretical and effective methane production. Some basic economic considerations were drawn to complete the study, considering actual management costs and seagrass production in the analysed geographic area, to propose an alternative managing route, having positive environmental and economic aspects. The possibility of using beach-cast residue after AD as solid digestate in agriculture was investigated by means of heavy metal characterization.

4.5. Materials and methods

4.5.1. Study area and sampling

Beached seagrass was collected along the coastline of Friuli Venezia Giulia, North-East of Italy (municipality of Grado, Adriatic Sea, North-Western Mediterranean).

The seagrass meadow was subdivided in two parts and was composed of different patches of *Cymodocea nodosa*, *Zostera noltii* and *Zostera marina.* The upper limit was close to the coastline and the meadow presented a high density up to 3.5 m of water depth, with a medium coverage to the bathymetry of 6-7 m.

The fieldwork was carried out in Spring 2019 and seagrass green wrack samples, with no visual signs of decomposition, were collected and transported to the laboratory without delay. The plant material was collected by random point sampling to avoid local effects on substrate properties. 30 sampling points were selected using global positioning system (GPS) tracking and about 5-10 kg of beached residues were collected in each sampling point every week for the whole campaign. Seagrasses were isolated from the residual material; each wrack sample and leaves were cleaned by epiphytes and then were determined to species level. A part of the collected material was dried in an oven at 60° C for 24 h for evaluating elemental composition, moisture, hemicellulose, cellulose and lignin content.

4.5.2. Analytical methods

The analysed physicochemical parameters were elemental composition (C, H, N, O), pH, moisture, total solids (TS), VS, ash, hemicellulose, cellulose and lignin. All the analyses were conducted in triplicate and mean data were reported in the following. C, H, N fractions were determined using a Vario Micro Cube Elementar®, composed of a high-temperature combustion chamber (furnace) with oxygen-jet injection, a gas separation unit (temperature programmed desorption trapping column) and thermo-conductivity detector. Elemental detection limit was <50 ppm. O concentration was calculated as difference from the sum of the other elements.

Weende method (ANFOR, 1981) was used to analyse organic components (i.e. proximate composition) of seagrass wrack, as well as moisture, TS, VS and ash contents. Hemicellulose, cellulose and lignin content was determined according to the neutral detergent fibre (NDF), acid detergent fibre and lignin (ADF/ADL) analyses as described by Van Soest et al. (1991).

4.5.3. Theoretical models

The models that were considered in the following were elemental composition model (Buswell equation) and two component composition models, based on proximate analysis results.

4.5.3.1. Elemental composition model

Organic matter composition significantly influences methane production in AD process (Leisteur et al., 2010, Cocozza et al., 2011b; Liu et al., 2019a). A simple method based on elemental composition (Buswell equation) was developed to predict the theoretical methane potential of a generic substrate using a stoichiometric equation based on feedstock elemental composition (C, H, O, N) (Buswell and Mueller, 1952).

$$TMP\ (L\ CH_4/\ g\ VS) = \frac{22.4 *\ (a/2 + b/8 - c/4 - 3d/8)}{12.017 * a + 1.0079 * b + 15.999 * c + 14.0067 * d} \tag{1}$$

In Equation (1), TMP represents total methane potential, while the coefficients *a, b, c, d* are constants of each element, equal to the ultimate analysis-based mass divided by the element molar mass: a = mass/C molar mass = mass/12.0107; b = mass/H molar mass = mass/1.0079; c = mass/O molar mass = mass/15.999; d = mass/N molar mass = mass/14.0067.

Buswell equation assumes that the reported elements are the only components of the organic substrate, as well as a total biodegradability of the substrate, that can be converted into methane without ashes accumulation (Boyle, 1976). Moreover, constant temperature, perfect mixing and optimal bacterial conditions in the digester are assumed (Feng et al., 2013). By avoiding including substrate biodegradability, it is expected that the method should overestimate real methane production.

4.5.3.2. Component composition models

During anaerobic decomposition, the most soluble components (simple carbohydrates, amino acids, lipids) are easily degraded, while complex and high molecular mass molecules (hemicellulose and lignin) require a long hydrolysis time (Angelidaki and Sanders, 2004). Several models were developed in literature based on organic matter composition to predict methane production in AD from a given substrate. Rodrigues et al. (2019) developed several multivariate regression models for BMP prediction from plants and vegetables and considered different parameters and predictive variables. Two models best fitted BMP results and were consequently applied to the current tests. In the proposed models all the variables were expressed as g/kg VS. The expression for model 1, based on crude protein (PT), carbohydrates (CRB) and crude fat (CF), was reported in Equation (2):

$$BMP\ (L\ CH_4/\ g\ VS) = 115.302 + 9.371 \cdot 10^{-1} \cdot PT + 2.379 \cdot 10^{-1} \cdot CRB + 5.706 \cdot 10^{-4} \cdot LP^2 - 1.505 \cdot 10^{-3} \cdot PT \cdot CRB$$

(2)

Model 2, instead, was based on a higher number of parameters, including carbohydrates (CRB), crude fat (CF), crude protein (PT), lignin (LG), acid detergent fibre (ADF) (Equation (3)):

$$BMP\ (L\ CH_4/\ g\ VS)$$
$$= 108.888 + 8.064 \cdot 10^{-1} \cdot PT + 5.248 \cdot 10^{-1} \cdot CRB + 2.469 \cdot 10^{-4} \cdot LP^2 - 1.483 \cdot 10^{-3} \cdot PT \cdot CRB - 9.440 \cdot 10^{-4} \cdot CRB \cdot ADF + 2.223 \cdot 10^{-3} \cdot LG \cdot ADF + 3.740 \cdot 10^{-4} \cdot ADF^2$$

(3)

4.5.4. BMP tests

BMP tests were conducted using Automatic Methane Production Test System (AMPTS, Bioprocess). AMPTS was composed of 15 reactors, having a volume of 650 mL each. Inoculum and substrate were introduced in the reactors at the desired I/S ratio and the material was intermittently (30 s on-30 s off) mixed by a slow rotating (80% of maximum speed, 140 rpm) agitator (Figure 14). The reactors were put in a thermostatic water bath (dimensions 50x30x15 cm), maintained at a mesophilic temperature of 35°C. The incubation unit had power consumption of 1,300 W and maximum operating temperature of 100 °C (precision of 0.2 °C). The biogas produced in each reactor passed through a 3M NaOH individual vial (100 mL volume, dimensions 44x30x6 cm, fixing efficiency>98 %), that removed acidic gases (mainly CO_2), while CH_4 passed to gas monitoring unit. The gas measuring device (dimensions 51x44x18 cm) exploited liquid displacement and buoyancy principles and was composed of a multi-flow cell arrangement (mean power consumption of 15 W): a digital pulse was generated when a defined volume of gas (10 mL) flew through the device; the data were recorded and analysed by an integrated embedded data acquisition system. Detection capacity was up to 13 L of cumulative gas per channel for each batch test, while measuring range for instant gas flow rate was 10-120 mL/min. Accuracy and precision values furnished by the supplier were respectively 5% and 1%.

Three replicates were made for each test. The main input parameters for each set of tests, including inoculum and substrate amount, as well as I/S ratio, were summarized in Table 2.

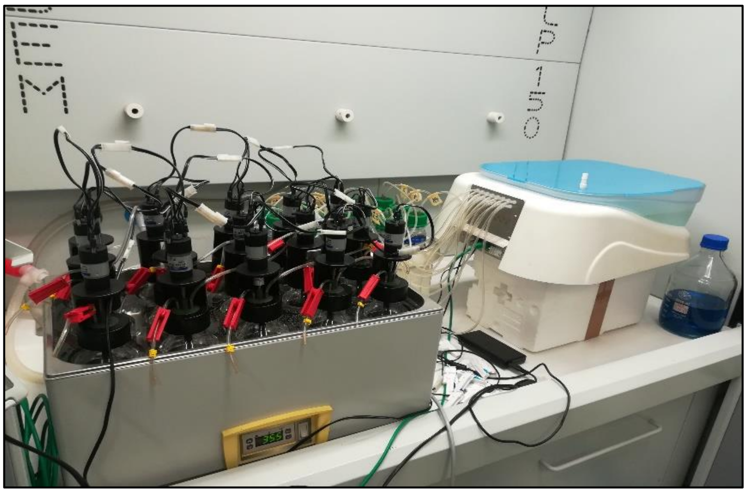


Figure 14: AMPTS equipment (Bioprocess Control)

Mesophilic anaerobic digestion sludge, taken from a full-scale traditional batch digester, located in Udine WWTP (North-east of Italy), was used as inoculum. A wide I/S ratio range of 2-13 was applied throughout the tests. Dried and wet material (fresh and dry seagrass washed in sterilized seawater) was tested to establish moisture effect on AD process. Before starting the tests, each reactor was flushed with N2 for 30 s to establish full anaerobic conditions (as suggested by Koch et al., 2015).

Table 2: Input parameters for BMP tests

Test	Substrate amount (g VS)	Inoculum amount (g VS)	I/S ratio
IS3 TQ	1.86 g fresh seagrass wrack washed in sterilized seawater	5.6	3
IS13 TQ	0.43 g fresh seagrass wrack washed in sterilized seawater	5.8	13
IS2 SS	2.88 g dry seagrass wrack	5.6	2
IS3 SS	1.92 g dry seagrass wrack	5.8	3
IS7 SS	0.83 g dry seagrass wrack	5.8	7
IS7 SU	0.84 g dry seagrass wrack washed in sterilized seawater	5.8	7
IS10 SU	0.56 g dry seagrass wrack washed in sterilized seawater	5.8	10

4.5.5. Heavy metal analysis

After each BMP test was completed, the digestate was separated from the supernatant and analysed for heavy metal (Cd, Cr, Hg, Ni, Pb, Cu, Zn) concentration. The organic matter was over-dried in stove at 105°C for 24 h and grinded. 0.2 g of samples were digested using 1 mL of 30% H_2O_2 and 9 mL of 65% HNO_3 according to the USEPA 3051a method (US Environmental Protection Agency, 1998). The samples were pre-treated in a microwave oven (CEM, MARS5 Xpress) and the extracts were filtered (0.45 µm PTFE), diluted and analysed. The elements were determined using an ICP-OES (Varian INC., Vista MPX) and multi element 36 ICP, with the addition of Hg standard, was used as internal standard.

4.5.6. Statistical analysis

Significance of observed differences in methane production were examined by one-way analysis of variance (ANOVA; significance level α=0.05) using MS Excel software. Statistical differences in methane yields were calculated between experimental and calculated values. When the calculated p-value was less than α and the F tested was major than F critical, the null hypothesis was rejected and consequently there were significant differences in analysed parameters. In the latter case, to conduct pairwise comparisons, Tukey test was calculated at α=0.05 and α=0.01: when the difference was greater than calculated values, it was considered as significant.

4.5.7. Simplified energy analysis

In order to evaluate the economic feasibility of plant upgrading to the combined treatment of sludge and seagrass, the actual landfill disposal costs (80 €/t) were considered, together with the amount of produced material. A significant variability in seagrass production emerged in the years 2004 – 2018, and this broad range was considered for the basic economic analysis. Seagrass production data were given from the beach managing company. A beach length of about 1.6 km was considered.

It was supposed to transport the material from the shore directly to the analysed WWTP (potentiality of 100,000 population equivalent, with a strong seasonal variability), where enough place was available to store the material before AD. The analysed digester, treating excess sludge, had a volume of 2,200 m^3 and was running only during the warm season (May-September), while it was stopped during the rest of the year, due to a substantial lack of substrate. The feasibility of continuously operating the digester was assessed, considering a mean hydraulic retention time (HRT) of beached seagrass of 25 days, consistent with BMP test results. Mean VS content of the material from physicochemical characterization and maximum energy yield from BMP tests were considered to evaluate additional electricity and heat production in the combined heat and power (CHP) downstream unit, having 36% electric yield and 45% thermal yield. Digester characteristics were given from plant managing company. Methane calorific value was estimated as 10.97 kWh/Nm^3 and the revenue from electricity selling was calculated using a mean valorisation rate of 0.20 €/kWh, coherent with Italian market. Heat production was not considered as revenue.

4.6. Results and discussion

4.6.1. Physicochemical characterization of seagrass biomass wrack

Considering the average values of all analysed samples, seagrass wrack organic matter contained about 45% of *Cymodocea nodosa*; in detail about 69% of organic matter was composed by leaves, while the residual 31% were rhizomes. Other two species were found in the seagrass wrack: *Zostera marina* (38.4 %, composed by 86.3% leaves and 13.9% rhizomes) and *Zostera noltii* (16.2%) with a greater amount of leaves (73.4%) rather than rhizomes (26.6%). The actual results were consistent with the data obtained in a previous sampling campaign carried out from years 2016 to 2018; a moderate variation (±10%) was detected in seagrass composition due to seasonal variability. After a bibliographic analysis and evaluation of seagrasses distribution maps in the study area, it emerged that there was a *C. nodosa* meadow, characterized by a wide density and coverage, which seemed to be in expansion both in the upper and lower limits. These outcomes supported the greater content of *C. nodosa* found in the current analysis, if compared to *Z. marina* and *Z. noltii*.

N content of seagrass wrack ranged from 2.4% to 3.1%, while H was 4.1-5.2%. C mean content was 35.7%; thus, mean calculated C/N and C/H ratios were respectively 12.8

and 7.4. A C/N ratio of 15-30 is recommended in AD process, to have an optimum microorganism activity (Xu et al., 2018); a low C/N ratio stimulates NH_3 build-up, while excessive C/N increases volatile fatty acids concentration (Siddique and Wahid, 2018). The obtained C/N ratio seemed favourable for AD application. Actual C content in the seagrass material was similar to what previously reported by Mateo et al. (2003) and Chiodo et al. (2016) in *P. oceanica*. Calculated O was 56.71% and was comparable to reported by Conesa and Domene (2015).

The results of proximate analysis of seagrass wrack were summarised in Table 3. TS content and crude fibre composition were similar to that previously reported by Marquez et al. (2013).

Crude protein showed a higher value, if compared to bibliographic data, whereas crude fat concentration was lower than that reported by Marquez et al. (2013). Analysing the biochemical composition, holocellulose (cellulose + hemicellulose) constituted the major component (40.13%), while lignin content was significantly lower than that reported by Ncibi et al. (2009) in P. oceanica, boosting for anaerobic valorisation of the material.

Table 3: Proximate values and biochemical composition of seagrass wrack (FM: fresh matter; DM: dry matter)

Proximate test	**Value (%, S.D.)**
Moisture, FM	33.33 ± 9.37
Total solids (TS), FM	16.26 ± 0.30
Volatile solids (VS), DM	11.05 ± 0.09
Ash, DM	10.47 ± 3.65
Crude fat (CF), DM	0.02 ± 0.00
Crude protein (PT), DM	11.01 ± 0.17
Crude fibre (CFib), DM	52.99 ± 1.95
N-free extract (NfE), DM	9.25 ± 1.30
Carbohydrates (CRB), DM	62.24 ± 0.26
Lignin (LG), DM	2.39 ± 0.01
Cellulose, DM	24.13 ± 0.15
Hemicellulose, DM	16.00 ± 0.07

4.6.2. Theoretical methane potential of seagrass wrack

Theoretical methane potential obtained using stoichiometric model (Buswell equation) was 251.7 mL CH_4/g VS; as first approximation, this value could be taken as the maximum methane yield obtainable from the analysed substrate. The BMP values from Model 1 (PT, CRB, CF) and Model 2 (CRB, CF, PT, LG, ADF) were quite similar, respectively 115.4 mL CH_4/g VS and 109.1 mL CH_4/g VS. Although the difference between the results of Buswell equation and proposed models was large, the obtained values were comparable to estimated and measured methane potential of terrestrial and marine biomasses having similar physicochemical characteristics reported in literature

(Table 4). The large difference obtained between the models could be explained with the fact that Buswell equation did not consider substrate biodegradability, while the applied models were adjusted to real anaerobic trials to consider also substrate characteristics, that significantly influence microbial activity and methane yield.

Table 4: Estimated and measured methane potential of terrestrial ad marine feedstocks

Feedstock	**Estimated methane potential (mL CH_4/g VS)**	**Real methane production (mL CH_4/g VS)**	**References**
Maize – *Zea mays*	296 - 390	289 ± 86	Amon et al., 2007; Labatut et al., 2011
Barley - *Hordeum distichon*	229 – 360	290 ± 83	Weiland, 2003; Dinuccio et al., 2010
Algae - *Spirulina platensis*	319	290	Biller et al., 2012; Sumprasit et al., 2017

4.6.3. BMP tests

The BMP curves obtained experimentally for each set of tests were compared in Figure 15 with the predicted values from stoichiometric Buswell equation and selected models.

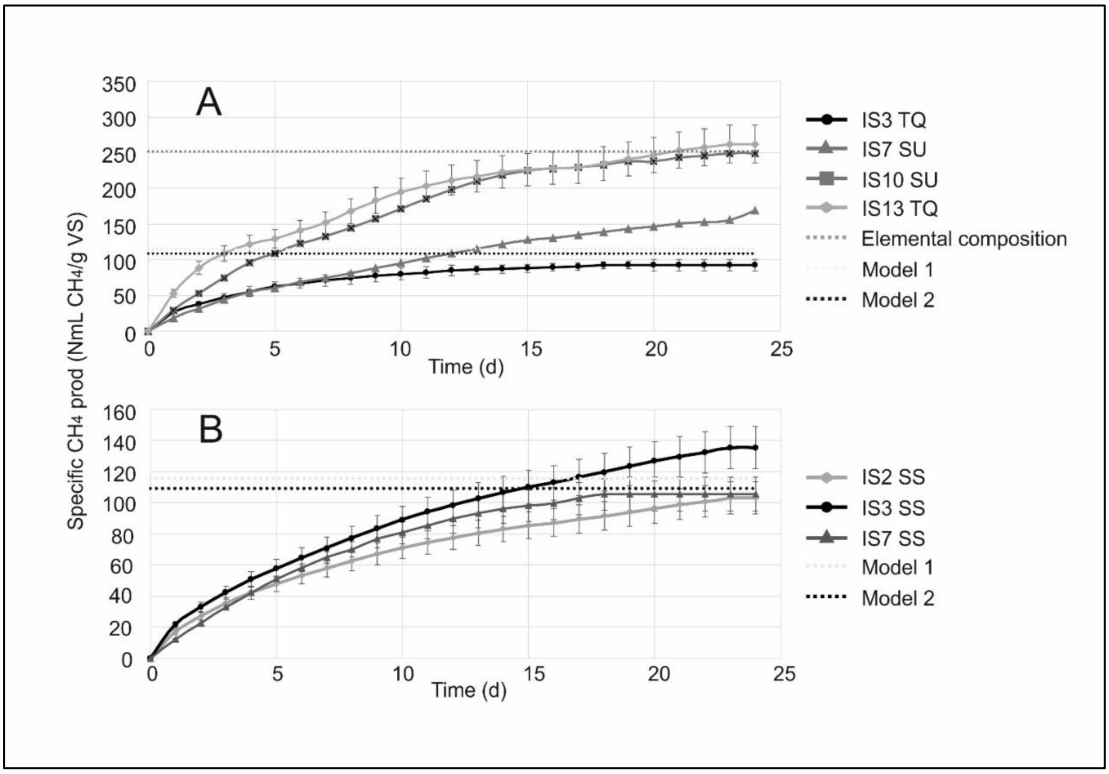


Figure 15: Measured and predicted methane yield in BMP tests. The horizontal lines represent predicted values using stoichiometric equation and selected models. A: CH_4 production from fresh seagrass wrack. B: CH_4 production from dry seagrass wrack.

In Figure 15A fresh seagrass tests were reported; CH_4 production significantly increased by increasing I/S ratio (in the range of 3-13). The maximum methane yield of 262.3 NmL CH_4/g VS was obtained at the highest I/S ratio of 13, and was about 3 times higher than that obtained at the lowest I/S ratio of 3 (92.5 NmL CH_4/g VS), indicating that a high I/S ratio is required to completely digest this substrate, probably due to its significant lignocellulosic fraction. Lignocellulosic material is slowly degradable, having a low hydrolysis rate, that is caused by its complex and compact structure (Li et al., 2018). Comparing theoretical methane potential from Buswell equation and the highest BMP value, yields were statistically similar ($P > p$ value; $P=0.063$).

Considering the methane yields from wet seagrass wrack, the maximum production was 249.4 NmL CH_4/g VS. Methane production from IS13 TQ and IS10 SU tests was not significantly different from statistical analysis; it could be stated that drying and subsequent rehydration processes had no significant influence on methane production, even if a higher I/S ratio was required for wet material to obtain the same methane yield. It was shown in literature that drying and rehydration of lignocellulosic material (sorghum grain) could even increase fermentability and digestibility (Brambillasca et al., 2019). The obtained methane yields were coherent with literature studies on lignocellulosic substrates: a CH_4 potential of 162.4 mL CH_4/g VS was highlighted in Liu et al. (2019b) from wheat straw after 25 d of digestion. Moreover, as shown in Table 3, similar yields were reported as for maize, barley and algae (Weiland, 2003; Amon et al., 2007; Dinuccio et al., 2010; Labatut et al., 2011; Biller et al., 2012; Sumprasit et al., 2017). Analysing more in detail BMP yield at the different applied I/S ratio, a strong linear relationship between I/S ratio and final methane yield appeared in a broad I/S range of 3-10 (R^2=0.9914): an increase of I/S ratio of 1 unity allowed to obtained an extra yield of about 22.2 NmL CH_4/g VS.

Methane production from dry seagrass (Figure 15B) was coherent with the values obtained from the applied models, with exception of IS3 TQ test, whose production was lower. A significant difference was highlighted between the methane yield from fresh and dry seagrass. The maximum methane yield from dry matter was 135.2 NmL CH_4/g VS applying an I/S ratio of 3 (Figure 15B). Considering an I/S ratio of 7, it could be seen that using wet material instead of dry seagrass increased CH_4 yield of about 60%; as for maximum BMP yield, instead, this increase was up to 94%. The present results were confirmed by previous studies (Nicastro et al., 2012; Liu et al., 2019a): Nicastro et al. (2012) reported that moisture could enhance soluble material loss via leaching, but also supply more suitable conditions for bacterial activity, accelerating hydrolysis phase.

Comparing theoretical methane potential from Models 1 and 2 with experimental results, only methane yields in IS7 SS test was considered significantly different ($p<0.05$), due to the reduced methane production. Analysing specific methane flux, CH_4 production peak always appeared in the first day of tests and was in a restricted range of 12.3-29.7 NmL CH_4/g VS d, a part from IS13 TQ test, where it was significantly higher (Table 5). The intense methane production during the first digestion day could be related to the seagrass wrack characteristics: Enríquez et al. (1993) and McMahon and Walker (1998) claimed that the presence of a lower C/N ratio and high initial N concentration promoted decomposition process. The conversion of highly degradable material in the initial digestion phase contributed to the consistent recorded methane production.

Table 5: Maximum daily methane production flux (NmL CH_4/VS d).

Test	Maximum methane production flux (NmL CH_4/g VS d)	Day of tests (d)
IS3 TQ	26.6	1
IS13 TQ	52.8	1
IS7 SU	18.8	1
IS10 SU	29.7	1
IS2 SS	16.9	1
IS3 SS	21.6	1
IS7 SS	12.3	1

4.6.4. Heavy metal presence in digestate

Heavy metal concentration in the digestate after AD process was reported in Table 6, together with the limit values of Italian legislation for agricultural use, represented by D. Lgs. 99/92 (Italian Government, 1992) and D. Lgs. 152/2006 (Italian Government, 2006).

Table 6: Heavy metal concentration in the digestate after AD.

Element	Limit value from Italian legislation (mg/kg DW)	Concentration in digestate I/S=3 (mg/kg DW)	Concentration in digestate I/S=7 (mg/kg DW)
Cd	<20	0.81	0.85
Cr	<750	2.44	2.85
Hg	<10	0.82	0.95
Ni	<300	7.21	8.41
Pb	<750	15.51	9.53
Cu	<1,000	88.82	47.68
Zn	<2,500	167.78	110.49

Detected concentrations were well below the limits for all the analysed metals. Cu and Zn presented the highest concentration if compared to the other heavy metals. Cd, Cr, Hg and Ni concentration was higher at I/S=7, suggesting that these elements were more concentrated in seagrasses rather than in sludge. Pb, Cu and Zn, instead, were more abundant in I/S=3 tests, due to the higher sludge concentration. As reported by Udayanga et al. (2018), coherently with the present results, Cu and Zn are frequently dominant in sludge, due to their use in water pipes, taps and galvanised materials. Considering the sewage sludge, predominant concentrations of Cu and Pb are present in the organic and sulphide or residual fractions, while Zn is mainly found in the Fe-Mn oxide or the organic and sulphide fractions (Udayanga et al., 2018). Cr and Ni have lower mobility and predominantly appear in the residual fraction (Udayanga et al., 2018).

Several studies reported seagrass capability to accumulate in roots and leaves trace elements presents in sediments (Bonanno and Di Martino, 2016; Díaz et al., 2017; Moustakas et al., 2017). Seagrass aboveground tissues accumulate higher Cd and Cu levels, whereas other metals, such as Pb and Zn, are mainly restricted to the belowground tissues (Hu et al., 2019). However, these heavy metals are generally more concentrated in common energy crops used for biogas production, rather than in the actual digestate (Abhilash et al., 2016; Knoop et al., 2018). Heavy metal presence in the analysed digestate after seagrass AD was not potentially harmful for agricultural reuse, representing a positive issue for AD application, also in co-digestion with sewage sludge. AD could be applied to recover energy in biogas form, avoiding landfill disposal and reducing transportation costs. In literature several studies proposed the agricultural reuse as soil improver of *Posidonia oceanica* residues (Cocozza et al., 2011a; Cocozza et al., 2011b).

4.6.5. Simplified energy analysis

The results of the simplified energy analysis were reported in Table 7.

The amount of beached seagrass transported to landfill varied consistently in the years 2004-2018, depending on local factors (such as climate and sea conditions), that favoured a higher or lower production. The yearly expense for landfill conferral varied in an analogous way, due to the constancy of specific disposal cost. The mean amount of seagrass transported to landfill was 3,472 t/y, corresponding to a seasonal disposal cost of about 277,800 €.

Table 7: Simplified energy analysis results.

Parameter	Value
Seagrass amount (t/y)	1,465-8,454
Disposal cost (€/y)	117,200-676,320
Seagrass VS amount (t VS/y)	161.9-934.2
Digester HRT (d)	25
Digester OLR (kg VS/m^{3}d)	1.84-2.36
Extra-days of digester operations (d)	40-180
Yearly methane production (Nm^3 CH_4/y)	42,413-244,752
Available energy (kW)	53.1-306.5
Electricity production (kW)	19.1-110.3
Heat production (kW)	23.9-137.9
Revenue from electricity selling (€/y)	33,500-193,300

Considering mean production, seagrass alone would contribute to allow the selected digester to operate for about 4 extra months, leading to a smoother operation throughout the year. In order to estimate organic loading rate (OLR), a mean HRT of 25 days was considered, consistent with the results of BMP tests and with the pilot-AD tests reported in De Sanctis et al. (2019), where AD of *Posidonia Oceanica* residues was studied. The upgrading of AD plant to the combined treatment of sewage sludge and seagrass would allow to operate the digester for about 9-10 months; increasing the amount of collected seagrass (for example by recovering the material for an extended beach length) would assure to cover the whole year. Considering the years when the highest seagrass production was recorded, however, continuous operations of the digester would be already assured. Mean methane production was evaluated as 100,526 Nm^3 CH_4/y, corresponding to a revenue from electricity selling of about 88,200 €/y. It is known that co-digestion of sewage sludge and complementary substrates, such as organic fraction of municipal solid waste (OFMSW) or fats, oils and greases (FOG), can lead to a higher process stability, reducing inhibition phenomena, together with an increased biogas yield (Sarpong et al., 2019; Cabbai et al., 2016), so a positive effect also on digester stability is expected after plant upgrade.

The obtained positive outcome from the energy analysis was due not only to an enhanced biogas production in AD process, but also to the possibility of applying the digestate to the soil, considering the low measured heavy metal concentration. The environmental outcome is undoubtedly positive, because of the significant reduction of transportation costs, given the proximity between the studied beach and the analysed WWTP, coupled with the avoiding of landfill conferral. Huge investment costs for plant upgrade would not be needed, given the presence of both an operating anaerobic digester (with auxiliary items) and a downstream co-generative unit, together with sufficient available volume to store seagrass before AD treatment.

This basic analysis should be supported by a further deepening at laboratory and pilot scale to evaluate the operating conditions of the digester in the new configuration and the possibility of co-digesting sewage sludge and beached seagrass, to plan the upgrade of full-scale AD plant. A successive pilot-study is forecast as prosecution of this work, to analyse the best applicable HRT and OLR to maximize methane yield, as well as to evaluate eventual troubleshooting that could arise in the system start-up. Moreover, the effect of material pre-treatment (such as mechanical grinding) should be evaluated, to enhance methane yields.

4.7. Conclusions

This study aimed at carrying out a preliminary evaluation of seagrass potential reuse as substrate for anaerobic digestion, considering that in the high-Adriatic coast all the material is actually transported in landfill, with huge environmental and economic costs. Methane yield from seagrass wrack was in the range of 103.1-262.3 NmL CH_4/g VS and was successfully modelled with Buswell equation and simplified regression models based on proximate analysis. Fresh seagrass wrack washed in sterilized seawater produced higher methane yields, if compared to dry substrate (up to +94 %), given the enhanced microorganism activity in moisture presence. Heavy metal concentration in the digestate was monitored to assess the feasibility of digestate agricultural reutilisation and it was shown that heavy metal concentration was well below the required legislation limits. A simplified energy analysis demonstrated that through anaerobic digestion the actual landfill cost of about 280,000 €/y would be avoided, and a mean income of 90,000 €/y would be obtained from electricity selling, allowing the selected digester to operate more continuously in the cold season. The obtained results boost for energy recovery from beached residues; a further study is required to analyse seagrass and sewage sludge co-digestion, underlining possible synergistic effects and evaluating the best operating conditions of the system, to achieve the highest methane yield.

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5. Environmental methane emissions from seagrass wrack and evaluation of salinity effect on microbial community composition using biochemical methane potential assays

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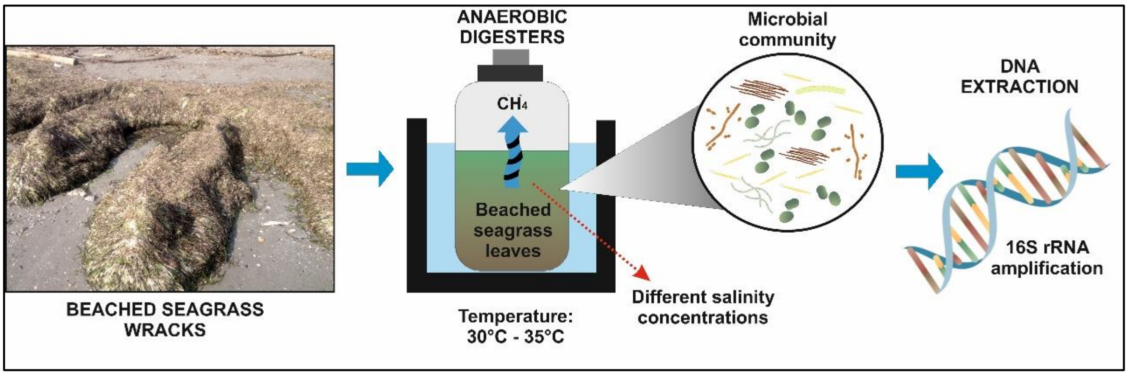
5.1 Abstract

In this work, methane emission from beach-cast seagrass, coming from the High-Adriatic coast, was analysed to evaluate the contribution to green-house gases emissions. Biochemical methane potential tests were used to evaluate CH_4 emission at different temperatures (30°C and 35°C) and different salinity levels in the seagrass wrack were tested. The changes in the microorganism community composition at the different salinity levels (from 0‰ to 35‰) were investigated, analysing the influence of the different species on methane production. The results underlined a specific CH_4 production from analysed seagrass in the range of 0.90-1.37 NmL CH_4/g VS d at 35°C and 0.36-0.50 NmL CH_4/g VS d at 30°C; the most intense methane generation was

observed at intermediate salinity levels of 18‰ at 35°C and 9‰ at 30°C. Total seasonal emission from beach-cast seagrass was estimated as 0.1256 mmol CH_4/m^2g. Microbial community analysis highlighted that *Rhodobacteraceae* was present in all samples and ranged from 8% to 25%. Lower salinity samples showed a prevalence of *Ruminococcaceae* (7-34%) and *Sphingomonadaceae* (5-19%), while *Petrotogaceae* (1-21%) and *Acholeplasmataceae* (6-14%) were more abundant in samples with 35‰ and 18‰ of salinity. The role of obtained isolates bacteria should be further explored in a subsequent work to better understand their role in methanogenesis and the contribution to greenhouse gases emission.

5.2. Graphical abstract



5.3. Keywords

Beach-cast litter; Seagrass; Salinity; BMP tests; Metatranscriptomics sequencing.

5.4. Introduction

Seagrasses are plants distributed worldwide and form meadows that carry out a primary role in the sea, defined as “ecosystem engineer” (Wright and Jones, 2006). Indeed, they considerably influence the physical, chemical and biological environment enough to provide significant benefits to coastal waters and beaches (Gutiérrez et al., 2011). Sediments stabilization and coasts and dunes protection are among the most important ecosystem services (Hemminga and Duarte, 2000; Barbier et al., 2011; Nordstrom et al., 2011): like many terrestrial plants, seagrasses lose their senescent leaves and a substantial fraction of this material (15-55%, but in some meadows up to 90%) is deposited along the coasts through joint action by currents, winds and tides (Cebrian and Duarte, 2001; Boudouresque et al., 2006; Short et al., 2007; Boudouresque et al., 2016; Macreadie et al. 2017). Seagrass wedge-shaped accumulations, also called banquettes, are the result of a dynamic process of accretion/destruction, as reported by Mateo al. (2003), and range from few centimetres to several metres of thickness. Their presence adds nutrients to beach ecosystem and protects the shoreline during costal storms period (Mateo et al., 2003; Del Vecchio et al., 2013). Especially in beaches of touristic interest, beach-cast leaf litter deposits are often removed for aesthetic reasons, but also due to the risk of fires and the generation of intense odours during degradation (Inamura et al. 2011; Parente et al., 2013; Corraini et al., 2018).

Throughout the decomposition process, a gradual impoverishment in organic matter occurs, especially regarding C, N and P, and this phenomenon favours a longer exposure of seagrass litter to oxidative processes: carbon is partially released from the seagrass litter into the atmosphere as CO_2 and CH_4, both considered greenhouse gases (Mateo et al., 2006; Couplan et al., 2007; Lavery et al., 2013; Banerjee et al., 2018). Recently Liu et al. (2019) explored the contribution of beach cast seagrass accumulation to greenhouse gases (GHG) emissions using an ultra-portable GHG analyser: throughout the experiment conducted in laboratory, reproducing environmental conditions, only CO_2 emissions were detected during wrack decomposition, while CH_4 flux was below the detectable limit. There is, therefore, a real difficulty to measure methane emissions from beached seagrass wrack during decomposition process; however, given the significantly higher global warming potential of CH_4, if compared to CO_2 (Grubert and Brandt, 2019), it is important to estimate also CH_4 flux to calculate the total contribution to GHG emissions.

Several factors influence GHG emissions from beached seagrass residues. The decomposition process operated by decomposers and detritivores is influenced by physicochemical and morphological features of organic matter; in particular, soluble materials are rapidly consumed, while recalcitrant components, such as lignin and lignocellulose, can persist for a long time in the environment (Blum and Mills, 1991; Lesteur et al., 2010). Moreover, the carbon loss within the seagrass tissue is influenced by the specific species: Liu et al. (2019) reported that *Zostera nigricaulis* is less degradable than *Amphibolis antartica*, due the major presence of lignin in the first specie. Seagrass wrack deposits along the shores are exposed to different atmospheric and marine agents that alter the organic matter (Jordà et al. 2012). In fact, the material accumulated along the beach area between high and low tide lines is subject to cycling between wet and dry conditions, which can facilitate or slow down GHG emissions (Coupland et al., 2007; Liu et al., 2017; Liu et al., 2019). Furthermore, climatic conditions influence GHG emissions; in lower latitudes increased decomposition rates are observed due to higher temperatures (Zhang et al., 2008). Moreover, hypoxic/anoxic conditions and banquette compactness affect material preservation, as reported by Mateo et al. (2003). There is a lack of knowledge about salinity effect on GHG emissions, particularly CH_4. This topic has been studied only in industrial saline wastewater treatment (Lefebvre and Moletta, 2006). From available literature, it is known that moderate salinity stimulates microbial growth (e.g.: calcium and magnesium are important for biological macromolecules synthesis), while high quantities (mainly NaCl) inhibit bacterial metabolism, due to salinity toxicity to methanogenic bacteria (McCarty and Smith, 1986; Deppenmeier et al., 1999; Soto et al., 1993; Chen et al., 2003; Chen et al., 2008).

BMP (Biochemical Methane Potential) assay is one of the mostly used approaches for studying GHG emissions, in particular methane. This technique analyses methane production from a generic substrate during an anaerobic decomposition process. Normally the substrate to be tested is mixed with a known quantity of inoculum to encourage and/or accelerate methane production (Holliger et al., 2016). During the experiment the system is kept at constant temperature between 30 and 50°C (Chae et al., 2008). BMP tests are sensitive to operating conditions (temperature, pH, agitation intensity, inoculum to substrate ratio, i.e. I/S), as well as to substrates characteristics (Mainardis et al., 2019). In the present tests, BMP tests were aimed at reproducing typical environmental conditions observed in the analysed beach during the Summer period, evaluating CH_4 emissions at different temperatures.

Microbes play an important role in ecosystems, as they provide basic nutrients to plants, are involved in nitrogen and carbon cycles and soil formation (Bell et al., 2005; Peter et al., 2011). Moreover, they represent a major portion of genetic diversity on Earth (Delgado-Baquerizo et al., 2016). Bacteria positive effects on terrestrial plants decomposition are well understood, but relatively little is known about the mechanism through which microbiomes degrade leaves and roots of seagrass and other aquatic plants. Furthermore, as previously mentioned, salinity effects on anoxic bacteria degradative capacity, and the subsequent consequences on microbial community, are still unclear. Omil et al. (1995) reported that there is an adaptation phenomenon of active methanogens to salinity level; Feijoo et al. (1995) reported that sodium toxicity depended on the type of substrate in which bacteria developed and the antagonistic effects among different ions concentrations.

Given this general framework, in this work biochemical methane potential (BMP) assays were used to estimate methane emissions from beach-cast seagrass wrack at different temperature levels. Salinity influence on methane emission, and the consequent contribution to GHG generation, was studied. The High- Adriatic coast (North-East of Italy) was selected as case-study; seagrass samples were collected and were physico-chemically characterized in the laboratory. Successively, anaerobic tests were conducted to establish specific CH_4 generation, reproducing typical environmental conditions observed in the studied area during the Summer season. Additionally, the changes in the relative microbial community abundance at the phylum and family level were investigated at different salinity concentrations, using metatranscriptomics sequencing, to evaluate the effect of this parameter on the specific community composition and consequently on CH_4 generation.

5.5. Materials and Methods

5.5.1. Seagrass biomass sampling and treatment

Seagrass wrack biomass was collected randomly along the coastline of Grado municipality (45° 40’ N; 13° 24’ E), in North-East of Italy (Adriatic Sea, North Western Mediterranean).

The fieldwork was carried out in Summer 2019 and only green seagrass leaves without signs of decomposition were collected. Seagrass was isolated from other beached biomass and the leaves were cleaned by epiphytes. The whole sample was divided in six portions. One fraction was used to determine leaves to species level; part of the biomass was dried at 60°C (and successively at 110°C and 550°C) to define moisture, total solids (TS) and volatile solids (VS) content. TS and VS contents were measured by standard analytical methods (APHA, 2012). Other five fractions were washed from the sand using sterilized water with different salinity content (35‰, 18‰, 9‰, 4‰ and 1‰). The salinity content was standardized in the laboratory by adding inorganic salt ions proportionally to the amount of washing water. Subsequently, seagrass leaves were let soak in five identical containers with the previously mentioned synthetic salinity concentrations; after 24 h, they were extracted and used for anaerobic tests.

5.5.2. Elemental analysis

Seagrass samples were rinsed with freshwater to reduce the salt content prior to drying. The washing procedure was performed stepwise in batch mode until reaching a salinity close to 1‰ in the rinsing water. Each sample was dried at 105°C for 24 h and subsequently the organic matter was crumbled in a grinder. The samples were treated in a ball mill to reduce the organic material as powder. CHN fractions were analysed by Vario Micro Cube Elementar® and oxygen percentage was calculated in approximate way as difference from the sum of CHN concentrations. Three different replicates were carried out for analysis and mean values were reported in the following.

5.5.3. CH_4 generation tests

Methane production from seagrass was assessed using the standardized BMP technique. The BMP experiment was carried out using Automatic Methane Production Test System (AMPTS, Bioprocess®). The apparatus consisted of 15 glass digesters with known volume (650 mL, with 250 mL headspace) immersed in a thermostatic bath. Each reactor was connected with an individual vial containing NaOH through an insulated pipe: during the anaerobic process, sodium hydroxide fixed acidic gases, principally CO_2, while CH_4 passed to gas registration unit. The produced methane was registered by a dedicated electronic system, formed by injection mould flow cells, inserted in a further water bath, containing metal pieces that opened up and registered every 10 mL volume of CH_4.

In this experiment, the beached material with different salinity level was inserted into the reactors in the same mass amount (expressed as g VS) without adding digestate or sewage sludge; moreover, no nitrogen was flushed at the starting of the tests, in order to make the experiment more similar to the typical environmental conditions, where an oxidising environment appears at the top of the pile and anoxic conditions arise in the inner stratus. Three replicates were made for each salinity value (35‰, 18‰, 9‰, 4‰ and 1‰); in each reactor, 45 g of wet organic matter were inserted. Two different temperatures were tested: in the first analysis, the thermostatic bath was set up at 30°C, while in the second assay a higher temperature of 35°C was chosen. These temperatures represented, respectively, the typical and maximum temperatures recorded in Grado beach during the hottest Summer days in the last 10 years (ARPA FVG Meteo, 2019). The contribution to GHG emissions was evaluated considering the global warming potential of CH_4 for 100-year horizon, estimated as 28 (IPCC, 2014).

5.5.4. DNA isolation, PCR amplification, library preparation and sequencing

Microbial community DNA was extracted from leaf tissues: a sample having 35‰ salinity (comparable to environmental conditions) was analysed before the anaerobic process, while other 15 samples (3 replicates per each tested salinity level) were tested after AD process, to evaluate the modification in microorganism community composition after the tests. DNA was extracted using QIAamp® Stool DNA (Qiagen Laboratories, Carlsbad, CA, USA) following manufacturer instructions.

After collection, samples were frozen at -20°C. Later, sample suspensions were heated at 70°C for two times to lyse bacterial cell membranes (especially Gram-positive bacteria, that are difficult to destroy). In centrifugate samples inhibition adsorption tablets were introduced to remove salts and other impurities that could alter DNA reading process. After purification, nucleic acids were quantified by absorbance- and fluorescence-based method using Qubit 4 Fluorometer™ (ThermoFisher Scientific™, ng/µL), and NanoDrop™ 2000c (Thermo Scientific™, ng/µL).

The bacterial 16S rRNA gene was amplified using primers 16S-341F CCTACGGGNGGCWGCAG and 16S-805R GACTACHVGGGTATCTAATCC. Libraries were sequenced in a MiSeq (Illumina, CA) in paired end with 300-bp read length.

5.5.5. Data analysis

Reads were de-multiplexed based on Illumina indexing system. Adapters were removed using cut-adapt (Martin, 2011). Dada2 (Callahan et al., 2016) was used to obtain an amplicon sequence variant (ASV). Compared to OTUs (Operational Taxonomic Units), ASV is a higher-resolution analysis, which records the number of times each exact amplicon sequence variant was observed in each sample. Each output sequence was assigned to relative taxonomy using naive Bayesian classifier method, considering a clustering at 97% identity.

Downstream analysis was performed using R (R Core Team, 2018). Alpha-diversity was estimated using Chao1 (Chao, 1984) and Shannon's diversity index (Shannon, 1948).

Clustering was performed using the R function heatmap.2 on the read counts, expressed as percentage of the total. Differential abundance of species was tested using the fitZIG function of metagenomeSeq package (Paulson et al., 2013).

5.6. Results and discussion

5.6.1. Beached seagrass characterization

Seagrass deposits extended in the beach area closest to the sea and presented reduced dimensions during the Summer period, due to the daily beach cleaning operations. Seagrass wrack material was dominated by *Cymodocea nodosa* (41.5%) followed by *Zostera marina* (38.3%) and *Nanozostera noltii* (20.2%). Considering each species, in Table 8 moisture, CHN and calculated O values were reported, expressed as percentage. The highest moisture content was found in *Zostera marina*, while *Nanozostera noltii* presented the lowest humidity. Elemental analysis showed that *N. noltii* had the highest C and N content, whereas *N. noltii* and *Z. marina* presented similar H values. Calculated O ranged in a very similar way between 51.05% and 54.25%. The obtained were comparable to those reported by Fourqurean and Schrlau (2003) and Marquez et al., (2013). Mean TS and VS content was 16.26 ± 0.25% and 11.05 ± 0.08%, respectively, even if some fluctuations were observed in the measuring campaign due to seasonal variability.

Table 8: Results of elemental analysis and moisture content

Specie	Moisture (% w/w)	C (% w/w)	H (% w/w)	N (% w/w)	O (% w/w)
Nanozostera noltii	83.31 ± 3.84	40.05 ± 0.21	6.40 ± 0.15	2.51 ±0.00	51.05
Zostera marina	87.10 ± 2.58	39.30 ± 0.28	6.43 ± 0.14	1.70 ± 0.00	52.57
Cymodocea nodosa	84.09 ± 1.62	37.50 ± 0.42	5.85 ± 0.35	2.40 ± 0.00	54.25

The high moisture content of the analysed seagrass (Table 1) could enhance material degradation: as reported by Nicastro et al. (2012), moisture can accelerate the decomposition of seagrass accumulations both on chemical (e.g. fostering the breaking of soluble compounds) and ecosystem level (e.g. facilitating decomposers activity).

Previous analyses have shown that analysed seagrass leaves presented a low lignin content (2.39 ± 0.01), compared data reported by Ncibi et al. (2009). As shown by Klap et al. (2000), relative lignin abundance varies with specie and type of tissue and is positively correlated with life span. The species considered in the present study had a life span shorter than other seagrasses, for example *Posidonia oceanica*, that is typically characterized by a higher lignin content. Analysing NDF (Neutral Detergent Fibre, 52.99 ± 2.09% DW), ADF (Acid Detergent Fibre, 36.99 ± 1.64% DW), cellulose (24.13

± 0.15% DW) and hemicellulose (16.00 ± 0.07% DW) content, seagrass samples presented values comparable with those reported by Trevathan-Tackett et al. (2017).

5.6.2. BMP tests

BMP test results were summarized in Table 9, as for the tests executed at 35°C, and Table 10, as for the tests at 30°C. Interestingly, it could be observed that the highest CH_4 emission was obtained at intermediate salinity levels of 18‰ (at 35°C) and 9‰ (at 30°C). From Table 9 a specific CH_4 peak up to 2.45 NmL CH_4/g VS d (salinity of 18‰) and 2.22 NmL CH_4/g VS (salinity of 35‰) appeared at 35°C; reducing environmental temperature at 30°C, specific CH_4 emission was more consistent at lower salinity levels, with a specific CH_4 peak up to 1.57 NmL CH_4/g VS d (at 9‰ salinity). A preliminary campaign of BMP tests executed at 25°C showed no detectable CH_4 emission, so it could be concluded that CH_4 generation had a strong temperature dependence and could be measured only if a certain threshold (between 25 and 30°C) was reached. Considering CH_4 global warming potential, the highest specific GHG emission at 35°C temperature was recorded at salinity concentrations of 18 and 35‰ (38.29 m^3 CO_{2eq}/t VS d and 36.57 m^3 CO_{2eq}/t VS d, respectively), while at 30°C the maximum GHG emission was registered at 9‰ of salinity (14.06 m^3 CO_{2eq}/t VS d).

Table 9: BMP test results at 35°C

Salinity (‰)	Specific CH_4 emission (NmL CH_4/g VS d)	Equivalent GHG emission (m^3 CO_{2eq}/t VS d)	Specific CH_4 peak (NmL CH_4/g VS d)
0	0.90	25.19	1.22
4	0.94	26.29	1.43
9	1.13	31.77	1.57
18	1.37	38.29	2.45
35	1.31	36.57	2.22

Table 10: BMP test results at 30°C

Salinity (‰)	Specific CH_4 emission (NmL CH_4/g VS d)	Equivalent GHG emission (m^3 CO_{2eq}/t VS)	Specific CH_4 peak (NmL CH_4/g VS d)
0	0.48	13.35	1.21
4	0.46	12.77	1.18
9	0.50	14.06	1.35
18	0.36	10.18	0.90
35	0.37	10.43	1.01

Methane production increase with operating temperature increase was reported in several studies. Chae et al. (2008) analysed swine manure AD and highlighted that maximum methane yield was obtained at 35°C while, at 30°C, CH_4 production was reduced by 3%, if compared to 35°C. Considering the actual results, methane production at 30°C was about 5% lower than acquired at 35°C, consistent with literature findings. These basic results suggested that temperature is a determining factor on anaerobic decomposition process, and consequently on atmospheric methane release from the beached material.

Salinity influence on methane emission at the tested temperatures was summarized in Figure 16. An increase in CH_4 emission in the range of 0.08-0.20 NmL CH_4/d g VS °C was observed for an increase of 1°C in environmental temperature; this specific increase was more pronounced at the highest salinity concentrations of 18‰ and 35‰, meaning that temperature had a noticeable influence in GHG emissions of beach-cast material, which has typically a high salinity value of about 35‰.

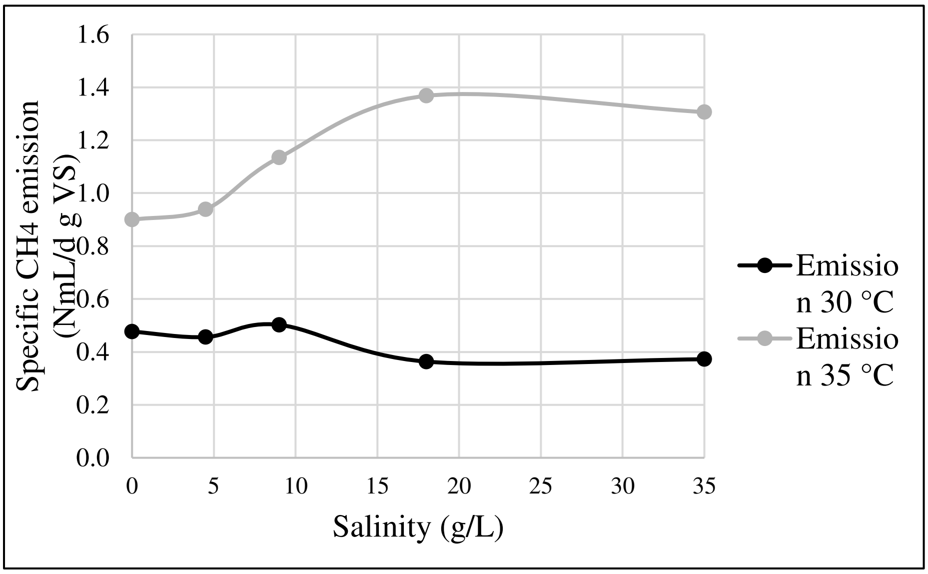


Figure 16: Salinity influence on specific methane emission at 30°C and 35°C

The present data are in line with that reported by Zhang et al. (2017): employing a BMP assay and using an acclimated inoculum, they concluded that at 35°C an increased methane production from marine macroalgae was obtained at salinity level below 35g/L, while the maximum methane yield was obtained at 15g/L salinity. In the current tests, the highest emission was achieved at salinity concentration of 18g/L, very similar to the value reported by Zhang et al. (2017). Several studies reported that a moderate salt concentration stimulates microbial growth, while high saline concentration (especially cations) slows down or even inhibits the anaerobic process (Soto et al., 1993; De Vrieze et al., 2018). This effect was not observed in the current results, because at 35°C temperature specific CH_4 emission was more consistent at the highest salinity concentrations (18‰ and 35‰), if compared to lower salinity levels (9‰, 0‰, and 4‰). This effect was attributed to antagonism phenomenon between Na^+, K^+, Mg^{2+} and Ca^{2+}. It was shown in literature that a balanced concentration of these cations increases sodium tolerance of methanogenic bacteria, generating beneficial effects on methane production (Soto et al., 1993; Yerkes et al., 1997; Hierholtzer et al., 2014). Zhang et al. (2017) showed that sodium, magnesium, calcium and potassium concentration equal to 1.53, 0.13, 0.18 and 0.03g/L, respectively, allowed to obtain the maximum CH_4 production.

Seasonal CH_4 emission from the analysed seagrass was estimated considering a total managed beach length of 1.6 km and mean local temperature data. In the year 2019, throughout the Summer season hourly soil temperature was higher than 29°C for 93 h. It was supposed, for successive calculations, that CH_4 was emitted only when environmental temperature reached 29°C. Total and specific CH_4 emission from the material was estimated considering biomass concentration (15kg/m^2) and using the laboratory emission data reported in Table 10. A mean retention time of the material on the shoreline of about 1.5d was considered (coherent with current cleaning operation procedures). Total seasonal emission was calculated as 0.1946 mmol CH_4/m^{2}d, higher than the values of 0.05-0.12 mmol CH_4/m^{2}d reported in Banerjee et al. (2018) and the values of 85.1 μmol CH_4/m^{2}d measured in Garcias-Bonet and Duarte (2017), probably due to different seagrass composition and variable climate conditions. Considering mean total seagrass production (3,472 t/y) in the managed area, total yearly CH_4 emission from seagrass material was estimated as 401.4 Nm^3 CH_4/y.

5.6.3. Microbial community composition

The distribution of the most abundant microbial families in all the analysed samples was shown in Figure 17. *Rhodobacteraceae* was detected in all the digesters and ranged from 8% to 25%. Also, *Synergistaceae* showed a good presence, with maximum values in the higher salinity samples (10-24%) and a significantly lower quantity in low salinity samples (2-20%). Conversely, samples with 4‰ and 0‰ of salinity showed a prevalence of *Ruminococcaceae* (7-34%) and *Sphingomonadaceae* (5-19%). *Petrotogaceae* and *Acholeplasmataceae* were present in greater quantity in samples with 35‰ and 18‰ of salinity, ranging from 1-21% and 6-14% respectively.

Table 11 shows the variation of indexes used to determine the alpha diversity in the analysed samples: Simpson diversity, Shannon's Index and Chao1 estimator were calculated. Simpson diversity Index measured the diversity among samples, intended as the number of species present in the sample and the relative abundance of each species. Values of Simpson diversity Index underlined that that analysed samples presented a discrete difference because the average value was close to 0.9, with a maximum for digesters 15, 10 and 14 (values: 0.9538, 0.9465, 0.9445, respectively) and a minimum for sample 3 (0.8839). Shannon's diversity Index allows to establish the biological diversity of ecological and microbiological communities. Considering the obtained values, the variation of Shannon's diversity Index showed the presence of a significant diversity in microbial association. Specie richness, intended as the number of species for each sample, was evaluated with Chao1 estimator.

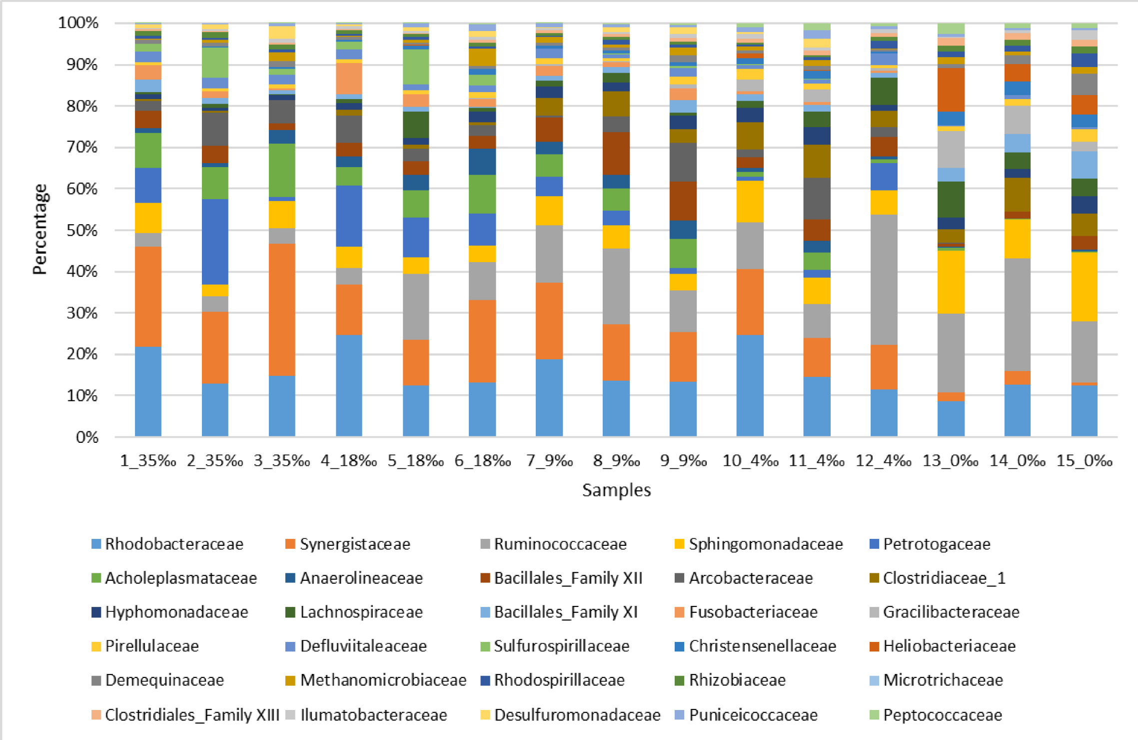


Figure 17: The 30 most abundant bacterial families detected in each sample after BMP tests.

This value depends on the number of reads present in the samples; as the number of reads increases, species richness is greater. Considering all samples, the number of species varied from 137 to 196, with a maximum value recorded in digesters 4 and 10 and a minimum value measured in samples 1 and 14.

Table 11: Simpson diversity, Shannon's Index and species richness (Chao1) in all samples

Sample (salinity, ‰)	Simpson diversity Index	Shannon's diversity Index	Species richness
1 (35‰)	0.9373	3.4971	146
2 (35‰)	0.9175	3.2405	160
3 (35‰)	0.8839	3.1777	159
4 (18‰)	0.9322	3.5735	196
5 (18‰)	0.9301	3.5189	177
6 (18‰)	0.9050	3.4446	174
7 (9‰)	0.9377	3.6811	174
8 (9‰)	0.9375	3.6439	159
9 (9‰)	0.9296	3.6826	170
10 (4‰)	0.9465	3.8884	181
11 (4‰)	0.9288	3.6973	151
12 (4‰)	0.9326	3.5595	163
13 (0‰)	0.9335	3.6088	153
14 (0‰)	0.9445	3.7195	137
15 (0‰)	0.9538	3.8309	167

Figure 18 shows the heatmap of digester samples clustered by Euclidean distance, computed basing on the 50 most abundant families. The cluster clearly separated high salinity (35‰ and 18‰) samples from low salinity (9‰, 4‰, 0‰) samples. An advanced test was carried out to identify the species responsible for this separation.

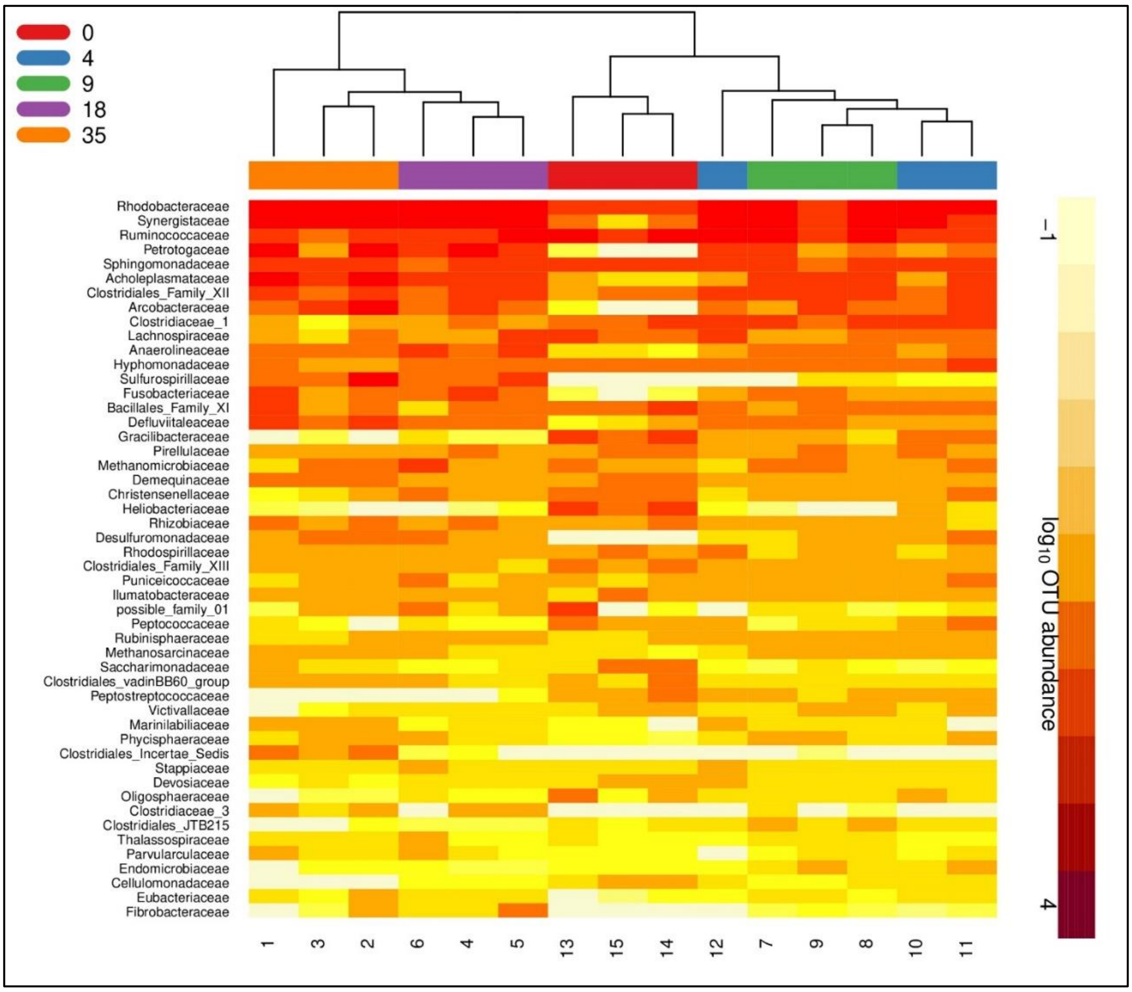


Figure 18: Clustering of samples based on the 50 most represented families: the colours on the top of the heatmap represent the five salinities; the colouring on the right of the heatmap is the abundance of OTUs in logarithmic scale.

Table 12 depicts the list of differentially abundant species between digesters with high and low salinity. All microbial families in samples with 9‰, 4‰ and 0‰ of salinity belonged to *Firmicutes* phyla, with higher and significant prevalence of *Ruminococcaceae* and *Clostridiaceae* families. Conversely, in digester with high salinity several anaerobic phyla were present, including *Proteobacteria* and *Fusibacteria* with a significant predominance of *Tenericutes* and *Thermotogae*.

Table 12**:** Differentially abundant OTUs between high salinity (35‰ and 18‰) and low salinity (9‰, 4‰, 0‰) samples.

Family	**Phyla**	**Reads in high salinity samples**	**Reads in low salinity samples**	**Adjusted p-value**	**$\log_2$ ratio**
Sulfurospirillaceae	*Proteobacteria*	3.3972	0.0741	0.0037	-1.7456
Acholeplasmataceae	*Tenericutes*	7.1435	2.4014	0.0221	-1.5172
Petrotogaceae	*Thermotogae*	9.2089	1.8352	0.0413	-1.5908
Fusobacteriaceae	*Fusobacteria*	2.7195	0.7827	0.0405	-0.9268
Clostridiaceae_1	*Firmicutes*	0.5593	4.7018	0.0001	1.8576
Peptostreptococcaceae	*Firmicutes*	0.0081	0.7173	0.0031	0.7358
Clostridiales Family XIII	*Firmicutes*	0.3802	0.9956	0.0037	0.5195
Ruminococcaceae	*Firmicutes*	5.7546	14.7681	0.0192	1.3002
Gracilibacteraceae	*Firmicutes*	0.0355	2.4325	0.0244	1.4269
Peptococcaceae	*Firmicutes*	0.0974	0.8350	0.0368	0.6465

Analysing the abundance of the first fifty microbial families, it emerged that most of them were related to strictly anaerobic or facultative anaerobic organisms. The most abundant family in all samples was *Rhodobacteraceae* (phylum: *Proteobacteria*), which are fundamentally aquatic bacteria that frequently thrive in marine environments and comprise mainly aerobic photo- and chemoheterotrophs, but also purple non-sulphur bacteria which perform photosynthesis in anaerobic environments (Pujalte et al., 2014). *Sphingomonadaceae* was another family abundantly found in the analysed samples and belongs to *Proteobacteria* phylum: they are ubiquitous bacteria and are commonly in soils, freshwater and marine habitats and activated sludge; members of this family are strictly aerobic chemoheterotrophs (Glaeser and Kämpfer, 2014). *Sulfurospirillaceae* (phylum: *Proteobacteria*) was the most significant family found in the digested high salinity samples. Bacteria belonging to this family are implicated in CO_2 fixation and are capable of autotrophic sulphide oxidation: Paul et al. (2017) showed that they are common in microbial benthic mat communities, in presence of the greenhouse gas methane.

One of the major phyla groups found after anaerobic digestion tests was *Firmicutes*, which are known to be involved in hydrolysing polymers (i.e. cellulose, lignin) and producing organic acids as metabolic endpoints (Lee et al., 2017). *Synergistaceae* in particular are important in bio-methane production: Baena et al. (2000) showed that their presence in digesters can increase methanogenesis process kinetic from 1% to 18%. This phylum generates organic acids that can be elaborated by other bacteria and produces acetic acid and hydrogen, that are utilized by methanogens (Fergurson et al., 2018). *Ruminococcaceae* and *Clostridiaceae* are another family of *Firmicutes* phylum that have the ability to break down complex carbohydrates. Some studies demonstrated that *Ruminococcaceae* and *Clostridiaceae* are associated with low methane production in anaerobic digestion process (Tian et al., 2014; Vanwonterghem et al., 2015): the present experiment confirmed the previous studies, because these families were found in samples with lower methane production (and also lower salinity).

Peptostreptococcaceae, *Gracilibacteraceae* and *Peptococcaceae* are other representative families (phylum: *Firmicutes*) that were found in low salinity samples: members of these families are obligated anaerobes and chemo-organotrophic and are generally found in different habitats including manure, soil and sediments (Slobodkin, 2014; Lee and Wiegel, 2015). In addition, recent studies highlighted that bacterial proteins (carbohydrate metabolism and transporter proteins) of *Peptococcaceae* and *Ruminococcaceae* were positively correlated with pH in digesters: a decrease in pH (i.e. during acidification) could be possibly detected by a decrease in metabolism of these families (Buettner et al., 2019).

Petrotogaceae family belongs to phylum *Thermotogae* and in this study it was significantly represented in high salinity samples. Buettner et al. (2019) demonstrated that *Petrotogaceae*, as *Synergistaceae* family, were positively correlated with temperature in anaerobic digesters, highlighting an ability to adapt to high temperatures.

Acholeplasmataceae (phylum: *Tenericutes*) and *Fusobacteriaceae* (phylum: *Fusobacteria*) were significant in samples with 35‰ and 18‰ salinity. The first are able to form acetic and propionic acid by glucose conversion (Martini et al., 2014), while the second are obligate anaerobic microorganisms that ferment carbohydrates or amino acids and peptides, producing various organic acids such as acetic, propionic, butyric, formic, or succinic acid (depending on the specific bacterium and substrate) (Olsen, 2014). Also, *Anaerolineaceae* were detected in the studied samples: in several studies they were isolated from anaerobic digester systems and it was determined that they present a fermentative metabolism, utilizing carbohydrates and proteinaceous carbon sources under anaerobic conditions (Sekiguchi et al., 2003; Sun et al., 2016).

Summarizing, microbial community analysis on digestate was fundamental to understand the dominant bacterial phyla active during methane production process.

Moreover, the results showed that microbial community underwent significant changes due to different salinity concentrations. This study demonstrated the microbial adaptability in the presence of environmental stresses, such as temperature and salinity increase. Furthermore, this approach has been useful to assess methane emissions from beached seagrass residues and the contribution to GHG emissions; however, this preliminary study needs to be deepened in a successive phase, applying similar tests on matrices having different seagrass composition, to extend the obtained results.

5.7. Conclusions

Biochemical Methane Potential tests were used to analyse the methane emission from beached seagrass wrack coming from the High-Adriatic coast at different temperatures (30°C and 35°C). The effect of different salinity concentrations on methane production was assessed, together with the modification in microbial community composition through DNA analysis in the digestate. The results proved that intermediate salinity levels (between 18g/L and 35g/L at 35°C temperature) produced the highest methane emission. CH_4 production from beached seagrass wracks was strongly dependent on environmental conditions (temperature and salinity), as well as on moisture level, which can accelerate material degradation. Methane emissions were strongly affected by microbial community composition: a greater microbial community diversity generally promoted methane production. The maximum gas amount was registered in samples where a high concentration of the phyla *Proteobacteria*, *Tenericutes*, *Thermotogae*, *Fusobacteria* and *Firmicutes* were present. In following studies, the role of obtained isolates bacteria should be explored to better understand the effect on methanogenesis and to analyse their environmental importance in greenhouse gases emission.

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6. General conclusions

Seagrass meadows are among the most productive ecosystems in the world, even though they cover less than 0.2% of the ocean floor and store about 10% of the carbon buried in the oceans each year. As terrestrial plants, seagrasses lose their leaves and these residues are transported on the surf zone where they form large litter wracks by the action of winds, currents and tides. Seagrasses piles can reach thicknesses from few centimetres to 2.5m. Seagrass litter wracks present considerable ecosystem services; however, they are removed in beaches of tourist interest. This organic material is often placed in accumulations near the beach or transported to landfill. This beach management presents several negative aspects such as great economic costs, GHGs emission increase due to seagrass wrack decomposition, use of non-renewable resources for litter transport to landfills and involves carbon loss from beach ecosystems. Therefore, a lack of synergy emerges between marine and terrestrial environments and management beach system. This PhD research was aimed at investigating the decomposition process of seagrass wracks in order to suggest an alternative reuse of this organic material. In detail, this study was focused on an energetic reuse of plant material using the anaerobic digestion process. The current PhD research led a major comprehension on the following aspects:

1. the decomposition process of beached seagrass residues is strongly regulated by environmental factors, such as salinity, temperature, water availability and solar radiation, and chemical-biological factors, as chemical characteristics of plants, species and presence of decomposers. A salinity concentration equal to 48 ± 1 g/l slowed down seagrass leaves degradation and this process was stopped with concentrations around 54 ± 2 g/l. Moreover, in natural environment seagrass decomposition was faster than in laboratory experimental setting, losing about 17% more organic biomass, due the

presence of other physical and biological factors that accelerated decomposition process.

2. It is possible to produce biogas through anaerobic digestion using seagrass litter as substratum and sewage sludge as inoculum. Preliminary results shown that methane yield from seagrass wrack was in the range of 103.1-262.3 NmL CH_4/g VS; this quantity was comparable to vegetable biomass actually used for biogas production. Moreover, biogas yield was successfully modelled with Buswell equation and simplified regression models based on proximate analysis. The quantity of produced methane depended on physico-chemical characteristics of seagrass and on organic matter moisture, indeed, the last factor greatly influenced the microbial activity: in litter with high humidity, methane production was greater than that produced by dry material. Moreover, further study is needed to evaluate the best operating conditions of the system, to achieve the highest methane yield.

3. Preliminary investigations have shown that it is possible to use the produced digestate by anaerobic digestion of seagrasses and sewage sludge in agriculture as fertilizer. Indeed, analyses carried out to determine heavy metals concentration in digestate shown that these concentrations were within the legal limits. However, further investigations are required in order to produce a fertilizer rich of all essential microelements indispensable for plants.

4. Focusing on the study area, a simplified energy analysis shown that through anaerobic digestion the actual landfill cost of about 280,000 €/y would be avoided, and a mean income of 90,000 €/y would be obtained from electricity selling, allowing the selected digester (that actually lack of substrate during the cold months, given the touristic characteristics of the area) to operate more continuously in the cold season. Moreover, anaerobic digestion plant treating sewage sludge and seagrass litter could operate for

about 9-10 months, suppling a continuous methane production with positive effects on digester stability.

5. Salinity concentrations and temperature had meaningful effects on methane yield. Methane production was higher of about 70% with an incubation temperature of 35°C, if compared to the quantity produced at 30°C. Moreover, the highest methane emission was produced at intermediate salinity level between 18 and 35 g/l at 35°C. Indeed, in presence of acclimated bacteria, moderate salt concentrations stimulate microbial growth, while low (< 9 g/l) and hight salinity (> 35 g/l) concentrations slow down or even inhibit the anaerobic digestion process. This effect was attributed to antagonism phenomenon between Na^+, K^+, Mg^{2+} and Ca^{2+} cations: a balanced concentration of these cations increases sodium tolerance of methanogenic bacteria, generating beneficial effects on methane production.

6. Focusing on the study area (1.6 km of length), total seasonal methane emission from seagrass wracks was estimated as 0.1946 mmol CH_4/m^2d, with a total yearly emission equal to 401.4 Nm^3 CH_4/y.

7. Analysing the microbial community, the maximum methane yield was obtained in samples where a high concentration of the phyla *Proteobacteria*, *Tenericutes*, *Thermotogae, Fusobacteria* and *Firmicutes* were present. Moreover, methane emissions were strongly influenced by microbial community composition. Indeed, a remarkable diversity of microorganisms promotes anaerobic activity.

In conclusion, due to a general EU perspective of further increase in renewable energy utilisation and energy demand increase, this study is a preliminary research for the reuse of seagrass litter residues aiming, therefore, at transform this organic material from waste to resource of clean energy (biomethane).

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