DOCTORAL SCHOOL IN CROP SCIENCE
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GREENHOUSE GAS EMISSIONS FROM CONSTRUCTED WETLANDS AND AGRONOMIC MANAGEMENT OF URBAN WASTEWATER AND DIGESTATE

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Riassunto

La consapevolezza e l'interesse verso l'impatto ambientale delle attività antropiche ha imposto la necessità di valutare in termini di emissione di gas ad effetto serra, oltre ai processi di produzione, anche i processi di gestione e smaltimento dei reflui prodotti.

Per il trattamento delle acque reflue la fitodepurazione, una tecnologia a ridotto impatto ambientale con scarsi o nulli input energetici, si sta sempre più diffondendo come sistema di trattamento naturale applicabile in vari contesti urbani e/o produttivi. Essa si basa sulla riproduzione dei processi fisici, chimici e biologici di autodepurazione del sistema suolo-piante-microrganismi che caratterizzano gli habitat acquatici e le zone umide naturali.

I processi depurativi, in larga parte operati dai microrganismi che si sviluppano nella rizosfera e che in questi sistemi contribuiscono alla riduzione del carico organico e azotato delle acque reflue, determinano il rilascio in atmosfera di diversi composti gassosi alcuni dei quali ad effetto serra, in particolare anidride carbonica (CO₂), metano (CH₄) e protossido di azoto (N₂O). La valutazione delle emissioni in atmosfera determinate da questi impianti, influenzate dalla tipologia impiantistica, dalla natura/tipologia dell'acqua reflua trattata e dalla presenza e specie vegetale impiegata, è studiata in Europa da circa 15 anni in impianti perlopiù siti nei Paesi del centro-nord, mentre poche sperimentazioni, e per lo più a scala di laboratorio, sono state condotte nell’area del Bacino del Mediterraneo; e con nessuno studio presso impianti di fitodepurazione Italiani.

In considerazione di quanto sopra, scopo principale del lavoro di tesi è stato quello di valutare il ruolo delle diverse componenti dei sistemi di fitodepurazione maggiormente diffusi in Italia (in scala reale o pilota) nelle emissioni di gas serra. A tale scopo sono stati scelti due siti situati in due differenti contesti bioclimatici italiani, Sicilia e Veneto, che trattano rispettivamente acque reflue urbane e frazione fluida di digestato. Particolare attenzione è stata rivolta al ruolo della componente vegetale del sistema sulle emissioni studiando differenti specie adatte alla fitodepurazione (Arundo donax L., Phragmites australis (Cav.) Trin. ex Steud., Cyperus papyrus L., Chrysopogon zizanioides (L.) Roberty eMiscanthus x giganteus Greef et Deu.).
Relativamente alle specie vegetali indagate, i risultati ottenuti nel contesto siciliano, hanno mostrato emissioni di CO\textsubscript{2} e CH\textsubscript{4} specie-specifiche con flussi di CO\textsubscript{2} significativamente maggiori (mediana 16.5 g m\textsuperscript{-2} d\textsuperscript{-1}) in presenza di A. donax, M. giganteus e P. australis, rispetto a C. papyrus e C. zizanioides e allo stesso letto non vegetato (mediana 5.2 g m\textsuperscript{-2} d\textsuperscript{-1}). L’impiego di M. giganteus e l’assenza di vegetazione hanno determinato emissioni significativamente maggiori di CH\textsubscript{4} rispetto a quelle monitorate con C. papyrus. Alla fine dei due anni di sperimentazione tutti i letti vegetati hanno mostrato un bilancio positivo della CO\textsubscript{2(eq)} con i valori più positivi calcolati per A. donax (21.4 kg CO\textsubscript{2(eq)} m\textsuperscript{-2}) mentre il sistema non vegetato ha mostrato una emissione netta in atmosfera di 5.5 kg CO\textsubscript{2(eq)} m\textsuperscript{-2}.

In Veneto, nell’impianto di fitodepurazione per il trattamento della frazione fluida del digestato proveniente da un impianto di digestione anaerobica di reflui zootecnici e colture dedicate, sebbene la P. australis e l’A. donax non hanno mostrato differenze significative nelle prestazioni depurative, quest’ultimo dopo lo sfalcio non ha ricacciato nel secondo anno di attività, determinando un incremento significativo nelle emissioni di CH\textsubscript{4} rispetto ai valori monitorati impiegando P. australis.

La frazione fluida di digestato, caratterizzata da un buon contenuto di sostanza organica e di azoto, può essere considerata anche come una risorsa da valorizzare mediante una sua gestione agronomica in un contesto dove la disponibilità di suolo non è un fattore limitante e tenuto conto dei limiti di sversamento imposti dalla Direttiva 91/676/CEE del 12 dicembre 1991. Infatti l’intensiva fertilizzazione minerale e le profonde lavorazione dei suoli agrari, caratteristiche dell’agricoltura italiana della seconda metà del secolo scorso, hanno determinato alcune criticità agli agro-ecosistemi, fra cui la perdita di carbonio organico. L’apporto di sostanza organica al suolo e l’impiego di tecniche agronomiche volte a ridurre le emissioni di CO\textsubscript{2}, sia direttamente che indirettamente, possono rappresentare una valida risposta alla perdita di carbonio organico con un effetto positivo anche sull’ambiente.

Tenuto conto di ciò, un ulteriore settore di indagine delle attività di ricerca del dottorato è stato quello di valutare l’effetto esercitato dall’applicazione della frazione fluida di digestato sulle emissioni di CO\textsubscript{2} da suolo agrario in relazione ai seguenti fattori: 1) dalla tessitura del suolo (franco sabbiosa vs franco argillosa) e dalle lavorazioni preparatorie
del terreno adottate (aratura vs rippatura) a seguito dello spandimento superficiale; 2) dalla profondità di interramento (10, 25 e 35 cm) a seguito dell’apporto al suolo del digestato tramite iniezione al suolo.

I risultati ottenuti hanno mostrato, con entrambe le metodologie di applicazione, un picco di emissione di CO$_2$ dopo un’ora dalla distribuzione ed emissioni che ritornano ai valori del suolo non ammendato dopo 3 giorni. Considerando la distribuzione in superficie, nelle due settimane successive allo spandimento, la tessitura franco sabbiosa ha determinato maggiori emissioni di CO$_2$ rispetto alla tessitura franco argillosa mentre nessun effetto significativo ha mostrato il tipo di lavorazione preparatoria del terreno. L’iniezione al suolo del digestato ha determinato nella prima ora post-distribuzione flussi di CO$_2$ in atmosfera con un andamento inverso alla profondità di interramento con minori emissioni al crescere della profondità.
Summary

The awareness of and interest in human activities environmental impact, in the framework of the ongoing climate change, has imposed the need to evaluate waste disposal in terms of greenhouse gases emission, in addition to the productive processes.

Constructed wetlands (CW) are a low environmental impact technology to treat wastewater with little or no energy input, increasingly used as a natural-like treatment system that is applicable in urban and/or production contexts. CW systems reproduce the physical, chemical and biological self-purification process of the soil-plant-microorganism systems that characterize aquatic habitats and natural wetlands.

Depuration processes, largely operated in these systems by rhizosphere microorganisms that contribute to the reduction of organic and nitrogen wastewater load, determine gaseous compounds release into the atmosphere, some of which act as greenhouse gases, in particular carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O). The evaluation of greenhouse gases (GHG) emission from CWs, influenced by CW and wastewater types and vegetation and species presence in the beds, has been investigated for about 15 years in CWs in central-northern European Countries, while few experiments, and mostly at laboratory scale, have been conducted in the Mediterranean Basin, and none in Italian CWs.

With this in mind, the main aim of this PhD thesis was to evaluate the role of the main components used in the construction of CWs on GHGs emission in the more widespread (full scale or pilot plants) Italian CW systems. For this purpose in two different Italian bioclimatic contexts, Sicily and Veneto, two CW sites were selected that treated urban wastewater and digestate fluid fraction respectively. Particular attention was paid in the research to the role of vegetation on CWs GHGs emission studying different species (Arundo donax L., Phragmites australis (Cav.) Trin. Ex Steud., Cyperus papyrus L., Chrysopogon zizanioides (L.) Roberty and Mischantus x giganteus Greef et Deu.).

The results obtained in the Sicilian context showed a species specific effect on CO₂ and CH₄ emissions. Significantly higher CO₂ emissions (median value 16.5 g m⁻² d⁻¹) were monitored in the beds vegetated with A. donax, M. giganteus and P. australis, than those vegetated with C. papyrus and C. zizanioides and the unvegetated bed (median value 5.2
g m$^{-2}$ d$^{-1}$). The *M. giganteus* presence in the bed and the absence of vegetation both determined significantly higher CH$_4$ emissions than those monitored with *C. papyrus*. At the end of the two trial years all vegetated beds showed a CO$_2$(eq) positive balance with better values calculated for *A. donax* (21.4 kg CO$_2$(eq) m$^{-2}$), whereas the unvegetated bed showed a net emission into the atmosphere of 5.5 kg CO$_2$(eq) m$^{-2}$.

The CW system in Veneto that treated digestate fluid fraction coming from an anaerobic digester for biogas production fed with livestock slurry and energy crops biomass, showed no significant depuration performance differences between *P. australis* and *A. donax* vegetation, but the latter did not regrow in the second year, thus determining a significant increase in CH$_4$ emissions.

The digestate fluid fraction, characterized by a high organic matter and nitrogen content, can also be considered as an agronomic resource in a region where land availability is not a limiting factor and considering the limit on its spreading imposed by Directive 91/676/EEC of 12 December 1991. In fact, the intensive mineral fertilization and deep soil tillage that were typical of Italian agriculture in the second half of last century, have caused some problems in the agro-ecosystems, including the loss of organic carbon. The addition of soil organic matter and the use of agricultural techniques to directly or indirectly reduce CO$_2$ emissions, may be a response to soil organic carbon loss with a positive effect on the environment.

Taking this into account, an additional investigation topic of the PhD research has been to evaluate the effect exerted by the digestate fluid fraction application on agricultural soil CO$_2$ emissions by: 1) soil texture (sandy loam vs. clay loam) and preparatory tillage (plowing vs ripping) after splash-plate spreading; 2) the digestate fluid fraction injection depth into the soil (10, 25 and 35 cm).

The results have shown a CO$_2$ emission peak one hour after digestate distribution and emission values reaching those of un-amended soils after 3 days, using both application methods. Considering the splash-plate technique in the two weeks following spreading, significantly higher CO$_2$ emissions were found in sandy loam than clay loam soil, the preparatory soil tillage showed no significant effect. Digestate fluid fraction soil injection determined after one hour of application, an opposite trend with injection depth, with lower emissions at increasing depth.
Chapter I

A review on the main affecting factors of greenhouse gas emission in constructed wetlands
Abstract

Constructed wetlands (CWs) are systems for wastewater treatment capable to remove both pollutants and nutrients without additional energy demand. In these systems gaseous compounds are release into the atmosphere through microbial processes. Among these gases carbon dioxide, methane and nitrous oxide are the most dangerous because they act as greenhouse gases (GHGs) and are well known as contributory factors to cause global warming. In this paper we reviewed 127 articles (from 1980 to 2014) from the scientific literature in order to analyze the most important factors that drive, in terms of quantity and type of GHGs, their production and emission from different CWs systems. Moreover wastewater flow and composition, feeding strategy, environmental conditions and plant species used to vegetate CWs have been considered.

Introduction

Constructed wetlands (CWs) are natural-like systems for wastewater treatment, which through various physical and biochemical mechanisms, based on substrate composition, microbes and plants ecosystems (Wang et al., 2008a, 2008b), are capable to reduce pollutants present in wastewaters (e.g. Heavy metals, Phosphorus, Nitrogen, etc) (Scholtz et al., 2007; Akratos et al., 2009) without additional energy demand (Inamori et al. 2007). To date the CWs are widely used to treat different types of wastewaters (Solano et al., 2004; Moir et al., 2005; Bulc, 2006; Borin and Tocchetto, 2007; Vymazal, 2009; Barbera et al., 2009; O’Geen et al., 2010; Verlicchi and Zambello, 2014) and during their pollutant abatement processes release gaseous compounds into the atmosphere. Among these gases carbon dioxide (CO$_2$), methane (CH$_4$), and nitrous oxide (N$_2$O) are the most dangerous for the environment because they act as greenhouse gases (GHGs) and are well known as contributing factors to cause global warming.

Already 20 years ago Nouchi et al. (1994) reported that CH$_4$ may be the dominant substance which will warm the earth’s surface. Thompson et al. (1992) reported that the global warming could be reduced by 25% if CH$_4$ emissions have been stabilized. Moreover N$_2$O emissions were reported not only to increase global warming, but also to destroy the UV atmosphere protection ozone layer (Dyominov and Zadorozhny, 2005).
Although the total GHGs emission from worldwide CWs view is lower compared to that from all other sources (natural wetland, agricultural soil, industry, ecc…) (Yan et al., 2012), the spread of this technology worldwide needs to understand its potential on GHGs impact. The quantity and type of GHGs emission from CWs are influenced by several factors including wastewater flow (Liu et al., 2009) and composition (Wu et al., 2009; Yan et al., 2012), feeding strategy (Jia et al., 2011), environmental conditions (Liikanen et al., 2006) and plant species used to vegetate this natural wastewater treatment systems (Wang et al., 2013). In this paper we reviewed 127 articles (from 1980 to 2014) to analyze in the literature the most important factors that drive the production and emission of GHGs from CWs.

The effect of CW type on GHG emissions

According Vymazal et al. (1998), CWs are classified considering the life form of the dominating macrophyte as: free-floating macrophyte-based systems, submerged macrophyte-based systems, and emergent macrophyte-based systems. These last systems, the most widely used, are further classified according their design in relation to the water flow in three major groups (Vymazal, 2007): free water surface flow (FWS); horizontal subsurface flow (HSSF); vertical subsurface flow (VSSF); and hybrid constructed wetland systems combining previous three types.

Teiter and Mander (2005) compared HSSF CWs vs VSSF CWs detecting slight higher emission of N₂O from VSSF (35.6-44.7 µg N₂O-N m⁻² h⁻¹) than from HSSF ones (4.4-19.5 µg N₂O-N m⁻² h⁻¹) since no significant differences were reported for CO₂ emissions. The CH₄ emission was higher in HSSF CWs than VSSF ones, and within the HSSF bed methane emission was significantly higher in the inlet zones (640-9715 µg CH₄-C m⁻² h⁻¹) than the outlet ones (30-770 µg CH₄-C m⁻² h⁻¹). Søvik et al. (2006) compared the N₂O, CH₄, and CO₂ fluxes in three CWs systems (HSSF – VSSF– FWS) located in North Europe (Estonia, Finland, Norway, and Poland) during winter and summer seasons. The average N₂O emissions were significantly higher in the VSSF one, for both summer and winter seasons (691.4 ± 33.0 and 8.2 ± 2.5 mg m⁻² d⁻¹ respectively) whereas the HSSF CW showed significant higher emission (5.4 times) than FWS ones only in the summer with 9.3 ± 1.3 mg N₂O m⁻² d⁻¹. No significant differences in CH₄ emission were assessed in summer among the three CWs; during the winter FWS CW
emitted significantly higher methane quantity (253.3 ± 112.0 mg m⁻² d⁻¹) than the other two CWs systems. The authors suggest that this higher emission might be due to an increased decay of C from plants or more anoxic condition due to ice cover. During summer, the CO₂ emission flux was significantly higher in VSSF CW (31,900.0 ± 7,700.0 mg m⁻² d⁻¹) than HSSF and FWS, in winter it was significantly lower in HSSF beds (2,530.0 ± 154.0 mg m⁻² d⁻¹) than in the other ones. Although the impact of the VSSF CW on Global Warming Potential (GWP) showed significantly higher CO₂ equivalents (CO₂(eq)) fluxes of N₂O and CH₄ than the other two CWs types, the authors reported that VSSF CWs however require lower surface area for the same water volumes treatment efficiency than HSSF and FWS. As a consequence the absolute effect on GHGs emission is probably lower than the other wetland systems which have similar GWP. In a life cycle assessment study, Fuchs et al. (2011) confirmed that VSSF CWs have less environmental impact per unit efficiency and construction equipment than HSSF CWs due to their better nitrogen removal and smaller footprint. Mander et al. (2008) measured CO₂, CH₄, N₂ and N₂O emissions in two CW systems: 1) a HSSF sand filter system vegetated by Phragmites australis and Scirpus sylvaticus, treating hospital wastewater; 2) a hybrid system treating raw municipal wastewater, consisting of a two VSSF beds (filled with limestone, ø 15–20 mm), planted with P. australis, a HSSF (filled with ø 5–10 mm crushed limestone mixed with gravel) planted with Typha latifolia and P. australis, and two FWS beds planted with T. latifolia. Comparing the HSSF and VSSF beds the authors reported no significant different N₂ emission (average median value of 0.16 g m⁻² d⁻¹). Liu et al. (2009) evaluated CH₄ and N₂O emissions comparing four pilot-plant CW systems: VSSF, FWS, HSSF and their combination VSSF-HSSF-FWS vegetated with P. australis and treating domestic wastewater with low C/N ratio (BOD₅:N ratio of 200:100). The authors reported that the FWS CW showed the highest tendency to emit CH₄ (36.6 mg m⁻² d⁻¹) and lowest N₂O flux (0.1 mg m⁻² d⁻¹); an opposite course was monitored in the VSSF CW with lowest CH₄ emissions, but highest N₂O flux (2.2 mg m⁻² d⁻¹). Considering the GWP the combined system showed lower environmental impact than other ones. Van der Zaag et al. (2010) monitored CH₄, N₂O, and CO₂ emissions comparing FWS and HSSF CWs vegetated with T. latifolia and loaded with dairy wastewater. FWS were built with two deep zones
and one shallow zone covering about 82% of the total area with 12-16 days of residence time. HSSF, with 18-22 days of residence time, was built with a 0.1 m layer of washed pea-stone overlaying a 0.65 m layer of washed gravel (Ø 20 mm and 38% porosity). The authors reported that FWS CWs presented significantly higher emissions of CH$_4$ and N$_2$O than HSSF CWs. In relation to wastewater depuration HSSF CWs removed as much or more C from the wastewater than FWS ones emitting less CH$_4$, N$_2$O emissions, in terms of N$_2$O-N emitted to N removed, ranging between 0.1% and 1.6% and are similar for both CW types. Considering the emissions as CO$_2$(eq) the ratio of N$_2$O to CH$_4$ emissions was nearly 1:1 in both CW types in agreement with Søvik and Kløve (2007) who reported approximately equal contribution to the GWP with an average value of 0.76 from FWS CW, polishing chemically treated municipal wastewater. Pan et al. (2011), in China, estimated life-cycle GHGs emission from a VSSF CW and conventional wastewater treatment plants concluding that to remove 1 kg of BOD, the CW system emitted only about the 50% of CO$_2$(eq) respect the conventional system. The study further suggested that countries like China should choose VSSF systems for decentralized wastewater treatment, which could also potentially reduce GHGs emission by 8–17 10$^6$ Mg y$^{-1}$ CO$_2$(eq) compared with the conventional centralized scenario. Zhao and Liu (2013) in a life cycle GHGs emission study, comparing the VSSF and a HSSF CWs in the city of Tianjin, China, added that the VSSF CW emitted about 50%, 36% and 39% lesser CO$_2$(eq) than HSSF ones respectively to treat 1.0 m$^3$ wastewater or remove 1.0 kg of COD or BOD.

Comparing two HSSF CWs beds substrate (coarse granitic and fine gravel) effect on CH$_4$ emission García et al. (2007) found that the differences in methane emissions rates were quite important, 0.055 and 0.073 mmol m$^{-2}$ h$^{-1}$ respectively for coarse and fine gravel. This result is not completely explained by the slightly greater efficiency of gravel HSSF, that removes 5–7% more COD than the other substrate, and probably involves a change in the rates of the biochemical reactions involved in organic matter (OM) removal linked to the different substrate porosity (Kadlec, 2003; Barbera et al., 2014a).

**Effect of wastewater origin and characteristics on GHG emissions**

Most of the early CW applications were for domestic and municipal wastewater although there are a growing number of applications dealing with animal and industrial
wastewaters, urban and agriculture stormwaters, mine waters, groundwater remediation, and other applications (Kadlec and Wallas, 2008).

**CO₂ and CH₄ emissions**

Wang et al. (2008b) compared the effect of two artificial domestic wastewaters (BOD of 50 mg L⁻¹, TN 15 mg L⁻¹ and TP 1.5 mg L⁻¹ or BOD of 100 mg L⁻¹, TN 30 mg L⁻¹ and TP 3.0 mg L⁻¹) on CH₄ emissions from VSSF CW systems unvegetated or vegetated with *Zizania latifolia*, *P. australis*, and *T. latifolia*. The higher gases emissions were measured under the higher pollutants loading. Particularly organic carbon load is an important factor that positively affects CH₄ emissions (Kadlec and Knight, 1996; Tanner, 1997; Søvik and Kløve, 2007; Corbella and Puigagut, 2014). García et al. (2007) underline that gas emissions also depend on the quality of the organic matter (OM) (for example the proportion of particulate and dissolved OM, or the amount of short chained fatty acids), and the CW site where measures are carried out.

Considering landfill leachate age, Chiemchaisri et al. (2009) detected a higher CO₂ and CH₄ emissions with young leachate (875.3 mg CO₂ m⁻² d⁻¹ and 124.7 mg CH₄ m⁻² d⁻¹) than older and stabilized one (615.0 mg CO₂ m⁻² d⁻¹ and 100.0 mg CH₄ m⁻² d⁻¹).

Using synthetic municipal wastewater with different influent C/N ratios in a VSSF CW Yan et al. (2012) reported a dramatical influence of this ratio on CO₂ and CH₄ fluxes. In fact, increasing C/N ratio from 2.5 to 10 by the C load, the CO₂ gas flux increased from 283.57±2.48 to 457.34±3.16 mg m⁻² h⁻¹. On the contrary, varying C/N ratio by enhance N quantity, CO₂ gas fluxes showed a decreasing trend of about 15%, from 466.97±3.85 to 396.59±1.38 mg m⁻² h⁻¹ whereas CH₄ emissions were negligible. Therefore the change of C/N ratio, as expected, has a different effect on gases emission in relation to the element used to modify it (C or N). The authors reported an optimal C/N ratio around 5 for simultaneously best biological nutrient removal and lowest GHGs flux. This indication was confirmed by Zhao et al. (2014) and Huang et al. (2014). Therefore the artificial control of C/N ratio in the CWs inlet wastewaters can determine a relatively high nutrient removal efficiency and, in the same time, a low GHG emission.

Stadmark and Leonardson (2005), in a laboratory study at 20 °C, showed that nitrate addition to wastewaters at concentrations of 8 and 16 mg L⁻¹ significantly inhibited CH₄ production, confirming the findings of Conrad (2002). On the contrary the highest CO₂
production was obtained at the highest nitrate concentration, which indicates that increased nitrate loading on ponds and wetlands stimulate organic matter decomposition rates (Paludan and Blicher-Mathiesen, 1996).

**N₂O emissions**

Zhou et al. (2008) monitored the N₂O emissions from a VSSF CW system filled with an Andosol, vegetated with *Oryza sativa* and fed with diluted digested liquid from a dairy cattle methane fermentation plant. The authors described higher N₂O flux (up to 188 mg N m⁻² d⁻¹) than literature available data probably due to lower dissolved organic carbon presence in the influent wastewater confirming the results of Bhandral et al. (2007). Wu et al. (2009) in FWS CWs beds filled with washed sand (particle size <2 mm) investigated the effect of different wastewater COD/N ratio (0:1, 2:1, 5:1, 10:1 and 20:1) on N₂O emission. The influent COD/N ratio induce significant difference in total N₂O emission; in fact the authors with a ratio of 20 reported an emission 10 times greater than others measured under the COD/N ratio of 5 and 10 which also determined lower emission than 0:1 and 2:1 ratio. Stabilization of COD/N ratio would be a relevant parameter to predict N₂O emission because affects biological nitrogen removal by nitrification and denitrification. The ratio of 7.1 COD/N ratio is required to achieve the total denitrification (Carrera et al., 2004). In addition to nitrate availability and redox status, C quantity is a considerable factor for denitrification process (Zhu and Sikora, 1995; Stelzer et al., 2014; Veraart et al., 2014), considering that N removal is mostly due to this process (Tanner et al., 2002; Morkved et al., 2005) with values, that in same cases, reach 89–96% of the removal N (Lin et al., 2002). COD/N ratio also affect temporal N₂O emission patterns with the 95.8% of the total emission concentrated in the first two days of wastewater treatment for COD/N ratio of 20 (Wu et al., 2009). Inamori et al. (2007), monitoring the N₂O emission under three different BOD concentrations (1000, 2000 and 4000 mg L⁻¹) in a vertical flow system, reported increasing N₂O emission rates in relation to higher BOD concentration. Inamori et al. (2008), comparing two influent wastewater loads (50 mg L⁻¹ BOD – 15 mg L⁻¹ TN – 1.5 mg L⁻¹ TP or 100 mg L⁻¹ BOD – 30 mg L⁻¹ TN – 3 mg L⁻¹ TP), found a N₂O flux ranging from 0 to 24.0 mg N₂O m⁻² d⁻¹ with low influent load and 0–52.8 mg N₂O m⁻² d⁻¹ with strong wastewater strength.
The effect of C source nature on the N\textsubscript{2}O production is less clear. Elefsiniotis and Wareham (2007) reported that denitrifying bacteria prefer, as carbon source, volatile fatty acids rather than organic complex molecules in agreement with Kozub and Liehr (1999) who reported that denitrification in the wetland was limited by the availability of easily degradable sources of organic carbon. However in disagree Adouani et al. (2010) showed that C source, molecular length, and type are not directly correlated to the amounts of N\textsubscript{2}O and/or NO produced.

N\textsubscript{2}O emissions from CWs are positively influenced by N load (Liikanen et al., 2006) and in particular by nitrate concentration (Stadmark and Leonardson, 2005) This result is in agree with Johansson et al. (2003) who also reported, during the summer season studies in FWS CW, a high atmospheric N\textsubscript{2}O consumption when the NO\textsubscript{3}\textsuperscript{-} water concentrations were mostly low, due to bacteria communities that use this gas, very hydrosoluble, for their metabolism. The greater atmospheric N\textsubscript{2}O consumption (78\%) was measured when NO\textsubscript{3}\textsuperscript{-} concentrations were below 0.5 mg L\textsuperscript{-1} with a negative linear correlation for nitrate concentration ranging from 1 to 6 mg L\textsuperscript{-1}. Gas consumption by bacteria may be linked to short age of electron acceptors (i.e. for the denitrifying bacteria nitrate deficiency) and, hence, they use N\textsubscript{2}O as a substitute of nitrate indirectly confirmed by the fact that NO\textsubscript{3}\textsuperscript{-} exert inhibitory effect on N\textsubscript{2}O reduction in denitrification pathway (Gaskell et al., 1981; Itokawa et al., 2001; Adouani et al., 2010). Alinsafi et al. (2008), in a laboratory scale experiment, studying the effect of COD/NO\textsubscript{3}-N ratio (3, 5 and 7) on N\textsubscript{2}O emission showed that the highest emission occurred at low ratio; also adding that COD/NO\textsubscript{3}-N could mainly increase the NO\textsubscript{2}\textsuperscript{-} accumulation which inhibits the N\textsubscript{2}O reductase leading to the N\textsubscript{2}O accumulation and emission. This process is due to the fact that the reduction rate of NO\textsubscript{2}\textsuperscript{-} was higher than that of N\textsubscript{2}O (Campos et al., 2009).

CWs N\textsubscript{2}O emission of course are also influenced by other chemical wastewater characteristics such as: 1) oxidation–reduction potential (ORP) that determine greater emission when treated wastewater are rich in NH\textsubscript{4}\textsuperscript{+}-N (Zhou et al., 2008); 2) pH that influence both N\textsubscript{2}O production and reduction (Van den Heuvel et al., 2011) as reported in Koskinen and Keeney (1982) who found higher N\textsubscript{2}O/N\textsubscript{2} ratios under low pH conditions whereas high N\textsubscript{2} production under alkaline conditions.
Therefore the $N_2O$ CWs emissions are the result of wastewater composition mainly COD/N ratio, $NO_3^-$ availability, ORP, pH and dissolved oxygen which affect nitrification and denitrification processes (Huang et al., 2013).

**Effect of feeding strategy on GHG emission from CWs**

Intermittent CWs bed loading, which mimics the pulsing hydrological regime of natural wetlands, is often used to increase pollutants abatement (Healy et al., 2007; Caselles-Osorio and Garcia, 2007; Lu et al., 2009; Mander et al., 2011) determining however different emission rate of GHGs.

In 10-year-old created riparian wetlands in the midwestern USA, Altor and Mitsch (2006) demonstrated significantly lower efflux of $CH_4$ in areas with a fluctuating water table with a periodically soil exposure to atmosphere compared to permanently inundated ones. It may be due to the more sensitive changes in sediment redox status of methanogenic communities than methanotrophs ones (Whalen and Reeburg, 2000).

Moore and Dalva, (1993) reported that $CH_4$ production is an anaerobic process and so high water table increase the production of $CH_4$ (Macdonald et al., 1998; Teiter and Mander, 2005; Mander et al., 2010). On the other hand, $N_2O$ emission is greater with low water table due to the increase in nitrification activity and in availability of $NO_3^-$ for denitrification (Martikainen et al., 1993; Reginaet al., 1996). In the same way $CO_2$ production increases when the water table falls, due to higher decomposition rates of OM in oxic conditions (Moore and Knowles, 1989; Silvola et al., 1996).

Jia et al. (2011) studied the effects of continuous and intermittent wastewaters feeding strategies on $N_2O$ emission from FWS and VSSF microcosm CWs vegetated with *P. australis*. The FWS CWs were flooded for 4 days and then dried for the next 3 days or kept flooded for the all week. Instead, VSSF CWs were saturated for 1 day and unsaturated for 2 days or kept saturated for 3 days. The $N_2O$ emissions from the FWS CWs were similar for both continuously and intermittently fed strategy ranging respectively from $0.17 \pm 0.04$ to $0.32 \pm 0.05$ mg m$^{-2}$ h$^{-1}$ and from $0.16 \pm 0.05$ to $0.31 \pm 0.03$ mg m$^{-2}$ h$^{-1}$. On the contrary the VSSF CWs $N_2O$ emissions were influenced by feeding strategies because they ranged from $0.17 \pm 0.06$ to $0.75 \pm 0.21$ mg m$^{-2}$ h$^{-1}$ with continuous fed and from $0.09 \pm 0.03$ to $7.33 \pm 1.49$ mg m$^{-2}$ h$^{-1}$ with intermitted fed strategy probably due to the more oxidant conditions in the beds that cause incomplete
denitrification with a release of N₂O as the end product instead of N₂ (Mander et al., 2014a). The percent of influent N converted to N₂O of the intermittently fed VSSF CWs was about 2.5% whereas for the other thesis was about 0.5%. In agreement Mander et al. (2011) studies on GHGs emission from a HSSF planted sand filter and from a hybrid treatment wetland system (VSSF and HSSF) planted filters characterized by intermittent hydrologic regime reported that, intermittent loading, enhanced significantly N₂O emission. Considering CH₄ emissions the authors found somewhat unpredictable results, in fact, under intermittent loading and higher water table fluctuations in HSSF CW bed, and so with unfavorable condition for CH₄ production, gas flux was 7–12 times higher than under more stable water table conditions. This can be due to CH₄ ebullition during a shift from a high to a low water table (Van der Nat and Middelburg, 2000).

Wang et al. (2014) to improve denitrification ability of VSSF CWs treating swine wastewater have tried a wastewater inlet from both inlet pipe and shunt pipe with four different ratio 1:0, 3:1, 2:1 and 1:1. The authors found the higher annual mean N₂O emission at the ratio 1:0 (31.24 ± 4.28 mg m⁻² h⁻¹) followed by ratio 3:1 (18.66 ± 3.71 mg m⁻² h⁻¹), 1:1 (11.39 ± 5.16 mg m⁻² h⁻¹) and 2:1 (8.91 ± 2.43 mg m⁻² h⁻¹). The ratio 2:1, in addition to determining the lower N₂O emission, had the best effect on treating swine wastewater, especially in terms of TN abatement (about 80%).

Although the feed strategy has not been much investigated compared to other factors discussed in this review the literature data agree with the result reported in Mander et al. (2014b) who found significant higher CO₂ and N₂O emission under low water table whereas higher CH₄ emission under high water table.

**Effect of environmental conditions on GHG emissions**

Environmental conditions greatly affect the dynamics of GHGs emitted from CWs influencing directly and indirectly both the plants and the heterotrophic microbial activities (Liikanen et al., 2006; Zhu et al., 2007; Wang et al., 2013).

**Temperature**

Taking into account the environmental temperature, Barbera et al. (2014b) and Maucieri et al. (2014a) reported a positive correlation between average air temperature and CO₂ emission from HSSF CWs vegetated with *Chrisopogon zizanioides* or *P. australis* and
with CO₂ and CH₄ emission from HSSF CW vegetated with *Cyperus papyrus* showing that environmental conditions should be linked to the plant species in the evaluation of CWs gases emission. Søvik et al. (2006) and Wang et al. (2008b) found a significant higher CH₄ emission in the summer compared with the other seasons confirming that CH₄ emission is greatly influenced by temperature. Moreover the plant photosynthesis and the heterotrophic microbial activities, that interact with CH₄ dynamic in the CWs, are both thermophilic processes and therefore, are dependent on seasonal temperature changes (Liikanen et al., 2006; Inamori et al., 2007; Wang et al., 2013). Therefore, air temperature affects the CH₄ emissions influencing both the CH₄-oxidizing and CH₄-producing microbial communities and their level of activity (Moore and Dalva, 1993). Considering specific substrate temperature it exert a positive effect on CH₄ emission (MacDonald et al., 1998; Kim et al., 1999; Johansson et al., 2004). The importance of temperature for both CH₄ production and consumption by microorganisms in the CWs was confirmed by Zhu et al. (2007), finding that under low temperature the substrates for methanogenesis are reduced and consequently CH₄ emission is dropped, with a greater effect in free water surface systems than HSSF CWs. The authors, as expected, always have measured a higher temperature in the HSSF CWs than the FWS CW. In fact the sub-surface flow systems frequently determine in the upper part of substrate, where the root system is higher developed (Barbera et al. 2014b; Maucieri et al. 2014a) and there is a litter accumulation, a mulching effect toward rapid temperature change that permit more buffer time for bacteria communities adaptation. Johansson et al. (2004) measured the flux of CH₄ from a pilot scale SFW CW reporting fluxes ranging from consumption of 375 mg m⁻² d⁻¹ to emission of 1739 mg CH₄ m⁻² d⁻¹. The authors monitored in summer season a CH₄ flux ca. 10-50 fold higher than other seasons reporting moreover that sediment and water temperatures accounted for a large proportion of variation in the CH₄ flux (33-43%). de Klein and van der Werf (2014) measuring CH₄ emission in the same month (May) in a SFW CW covered for 90% of its surface by *P. australis* (110 stems m⁻²) at two water temperatures, 15°C and 24°C, reported a gas emission respectively of 7.8 and 24.5 mg CH₄ m⁻² h⁻¹. Stadmark and Leonardson (2005) in constructed ponds receiving wastewater with different loads of NO₃⁻ reported that water temperature was a good predictor of CH₄ emission in all three
ponds with emissions between 1 and 54 mg m$^{-2}$ h$^{-1}$, when water temperature was higher than 15°C, and less than 0.6 mg CH$_4$ m$^{-2}$ h$^{-1}$ when water temperature was below 10 °C. Significant higher N$_2$O emission during the summer season than during the winter one, due to a slowdown of the denitrification and nitrification processes at low temperatures was found by Søvik et al. (2006). However Stadmark and Leonardson (2007) in a laboratory experiment using constructed pond sediments collected at different times of the year reported that N$_2$O and CH$_4$ production was different when incubated at identical temperatures (13°C or 20°C) and NO$_3^-$ concentrations indicating that there are other sediment parameters that are important for GHGs production potential. The authors also found that higher sediment incubation temperature resulted in higher CO$_2$ and N$_2$O production whereas CH$_4$ was not affected suggesting that CH$_4$ production was not directly inhibited by low temperature, but rather by a lack of available precursors at 13 °C.

Mander et al. (2010), although reporting a significantly higher release of N$_2$O, N$_2$ and CH$_4$ during the warmer period, didn’t find a specific significant correlation between the N$_2$O flux and water temperature.

**Solar radiation**

Significant correlation of CO$_2$ and CH$_4$ flow rates through the culms with solar radiations was reported by Picek et al. (2007) in HSSF CW vegetated with *P. australis*. Similarly, Barbera et al. (2014b) and Maucieri et al. (2014a) found a positive correlation between CH$_4$ emissions and solar radiation from HSSF CWs vegetated with *C. papyrus*, *C. zizanioides*, *Miscanthus giganteus*, *Arundo donax*, *P. australis* or unvegetated. Regarding CO$_2$, the correlation was obtained only for *C. papyrus*, suggesting that the plants play a significant role in mediating the GHGs emissions, as below described.

**Effect of plant species on GHG emissions**

The presence of macrophytes is one of the most conspicuous features of wetlands and their presence distinguishes CWs from unplanted soil filters or lagoons (Vymazal, 2011). The macrophytes growing in CWs have several properties in relation to the treatment process that make them an essential component of the design (Brix, 1997). Bigambo and Mayo (2005) demonstrated that microorganisms attachment on plants rhizosphere determined
higher nitrogen removal than plants uptake. Plants also enhance the settling of solids (Brix, 1997) and acts like a biological pump, converting sunlight into chemical energy and carrying oxygen from the aerial part to the root system (Gries et al., 1990; Armstrong et al., 1992; Brix et al., 1992; Jackson and Armstrong, 1999; Tanner, 2001; Sorrell and Hawes, 2010). Plants provide appropriate surface area and substrate (exudates) for microbial attachment and activity (Zhai et al., 2013), promoting the conversion of pollutants to CO₂ (oxidized zone), CH₄ (anoxic zone) and N₂O (both zones) (Inamori et al., 2007).

CH₄ and CO₂ emission

Several studies reported that GHGs flux from CWs is affected by presence of vascular plants (Picek et al., 2007). The GHGs flux tends to be species-specific, is influenced both by the phenology (Kim et al., 1999) and density of vegetation (Liikanen et al., 2006; de Klein and van der Werf, 2014). The total CO₂ and CH₄ flux emitted to the atmosphere in vascular plant-dominated natural or constructed wetlands is primarily mediated by three processes: gas bubbling and diffusion from sediments, and internal plant-mediated transport (Brix et al., 2001).

Faußer et al. (2013) measured oxygen partial pressures inside rhizomes of P. australis monitoring its diurnal dynamics and characterizing root-associated methane-oxidising bacteria biofilms. They found a diurnal oxygen partial pressure variation in the rhizomes (around 185 hPa from soon after sunrise to over mid-day and decreasing exponentially over night to about 80 hPa) and a densely bacteria presence on root surfaces, with 34–37% of this accounted for potential methane-oxidising bacteria. Furthermore considering that different plant species determine different root oxygen release (Brix et al., 1992) these results highlighted the indirectly plants species-specific effect on organic matter degradation and CH₄ oxidation due to the variation of biogeochemical processes involved in wastewater treatment.

Maltais-Landry et al. (2009a) reported that unplanted HSSF CWs units had higher CH₄ fluxes than planted ones, confirmed the species-specific effect and found lower emission with T. angustifolia than P. australis and P. arundinacea. The authors suggested that the oxygen transfer capacity among plant species varies, thus adequate plants selection could reduce methanogenesis. Therefore, macrophytes by influencing CWs microbial
processes can modify (increase or reduce) CH\(_4\) formation and oxidation and subsequently CH\(_4\) emissions (Wang et al., 2008b; Wang et al., 2013). The roots may alter the soil oxidation-reduction potential status due to the oxygen release which may increase CH\(_4\) consumption (Wang et al., 2013). The methanotrophs bacteria inhabiting the root zone can promote oxidation of around 20–50% of the CH\(_4\) produced (Schütz et al., 1989; Denier van der Gon and Neue, 1996). For freshwater wetlands it was reported that more than 80% of the diffusive CH\(_4\) could be oxidized in the oxic surface layer (Bosse et al., 1993; Conrad, 1996). Jacobs and Harrison (2014) investigated in a microcosm experiment the effects of \textit{Lemma} sp. on CH\(_4\) emission and did not find significantly different fluxes between areas with and without floating vegetation with an average value of 7.4±1.6 µmol m\(^{-2}\) h\(^{-1}\).

The results of Maltais-Landry et al. (2009a) confirmed the results obtained by Johansson et al. (2004) who reported, in FWS CWs, significantly higher CH\(_4\) emission from habitats dominated by \textit{Phalaris arundinacea} (318 mg m\(^{-2}\) d\(^{-1}\)) than areas dominated by \textit{T. latifolia} or \textit{Glyceria maxima} (both ca. 160 mg m\(^{-2}\) d\(^{-1}\)). In the same systems without emergent plants the authors recorded the highest CH\(_4\) flux rate (675 mg m\(^{-2}\) d\(^{-1}\)). Instead, Wang et al. (2013) reported a much higher CH\(_4\) flux in vegetated systems (69.8 mg CH\(_4\) m\(^{-2}\) h\(^{-1}\)) than unvegetated one (11.5 mg CH\(_4\) m\(^{-2}\) h\(^{-1}\)). No significant differences in CH\(_4\) emission between vegetated (\textit{Arundo donax} or \textit{P. australis}) and unvegetated HSSF pilot plant CWs, site in Mediterranean Basin were reported by Maucieri et al. (2014a). Wang et al. (2008b) studying unvegetated or monoculture (\textit{T. latifolia} or \textit{P. australis} or \textit{Z. latifolia}) VSSF CW systems found that the highest CH\(_4\) emission was reached with \textit{Z. latifolia} and unvegetated system showed lower emission than vegetated ones. Ström et al. (2007) comparing \textit{Juncus effusus}, \textit{T. latifolia} and \textit{P. australis}, reported that CH\(_4\) emission rate were affected by presence of vascular plants and tended to be species-specific. Wang et al. (2013), comparing two vegetated systems (monoculture and polyculture), monitored the highest flux of CH\(_4\) in the polyculture system (average total amount of CH\(_4\) released in the studied period 92.0 mg CH\(_4\) m\(^{-2}\) h\(^{-1}\)) with the peak emission in growth season and the lowest one in senescence. The authors reported that in polyculture system \textit{P. australis} and \textit{T. latifolia} affected the \textit{Z. latifolia} growth, resulting in higher CH\(_4\) emission. Zhu et al. (2007) monitored an increase in CH\(_4\) emission.
immediately after *P. communis* cutting in both FWS and HSSF systems, probably due to the rapid release of CH₄ retained inside the vascular systems of the plants stalks. The CH₄ flux remained for 15 days after plant cut higher in the plant harvest areas than un-cutting ones because the stalks still served as conduits for CWs bed gases because they are above the water surface. The flux dropped down when the stalk butts died and the root activity decreased due to the absence of further support by photosynthetic products. In the FWS after cut when plants were kept below the water surface, CH₄ flux remained lower in the cutting areas than un-cutting ones; and the only pathways left for CH₄ emission were bubbling and diffusion through flooding water. Considering that most of the diffusive CH₄ was oxidized, bubbling become the most important pathway. In agreement Barbera et al. (2014b) found an higher CH₄ emission after plant cutting from HSSF bed vegetated with *M. giganteus*. Therefore the choice of plants species and their management, which indirectly impact on rhizospheric microorganisms growth, influence CH₄ emission from CWs.

The CWs CH₄ flux rate is affected by different variables, but plants represent one of the most important factors influencing CH₄ flux to/from the atmosphere (Wang et al., 2013). Plant root exudates provide organic substrate for microorganisms (Zhu and Sikora, 1995; Ström et al., 2003, 2005; Picek et al., 2007). Macrophyte aerenchyma enables transportation of oxygen from the air to the belowground parts of macrophytes (Chanton et al., 1993; Rusch and Rennenberg, 1998; Laanbroek, 2010), and therefore, it plays an important role in the CH₄ emission rate. Liikanen et al. (2006) reported an emission of CH₄-C that was 25 times higher than the C reduction in the wastewater and so most of the C released as CH₄ was originated from the C produced within the wetland. In agreement Zhu et al. (2007) observed the maximum CH₄ emission at the maximum plants growth phase due to stimulation of methanogenic bacteria by root exudate (Nouchi et al., 1994). The differences in the macrophytes root and stem plant architecture, aerenchymous tissue, and oxygen availability for rhizospheric bacteria, affect the methanogens and methanotrophs biomass (Inamori et al., 2007). In VSSF CW units, Inamori et al. (2007), found that 90% of the root biomass was concentrated in the upper 10 cm of the bed vegetated with *Z. latifolia*, whereas the *P. australis* root was deeper and the biomass more evenly distributed from near soil surface to the bottom of
the bed. Furthermore the authors reported that the number of methanotrophs in *Z. latifolia* units, was greatest at a depth of 10 cm decreasing along the profile of the bed; on the contrary in *P. australis* units, a larger number of methanotrophs was observed at depths of 20 and 30 cm, and smaller number of these bacteria communities at the 10 cm soil depth following the root deepening in the CW substrate.

Although CO$_2$ is a major GHG, a limited number of studies are focusing on CO$_2$ emissions in CWs (Mander et al., 2014a) and only few of them compared plant presence and species effects. Vegetation presence (Ström et al., 2007) and plant species (Verville et al., 1998; Barbera et al., 2014b) had a significant impact on CO$_2$ emission, with higher CO$_2$ fluxes in planted CWs (Maucieri et al., 2014a; Maltais-Landry et al., 2009a). CO$_2$ fluxes associated with high plant biomass production (Liikanen et al., 2006; Barbera et al., 2014b) can be due to the increased rhizosphere bacterial activity in planted CWs (Gagnon et al., 2007) in relation to more labile carbon source due to plant exudates (Picek et al., 2007). In planted wetlands there is higher bacterial activity because rhizosphere contains several orders of magnitude more bacteria as compared to bare soil. The difference is mostly caused by the presence of bacteria attached to roots and rhizomes. The role of plants in mediating bacterial activity and CO$_2$ fluxes is illustrated by the seasonal decline observed in CO$_2$ fluxes of planted CWs at plants senesce, compared to the constant fluxes observed in unplanted units (Maltais-Landry et al., 2009a).

**N$_2$O emission**

Monitoring a FWS CW Johansson et al. (2003) detected higher N$_2$O emissions from the area vegetated with *P. arundinacea*, followed by areas with the presence of *L. minor*, *T. latifolia*, *Spirogyra* sp. and *G. maxima*. The authors also observed a consumption of atmospheric N$_2$O for about 15% in all measurements, detecting the highest consumption from “moderately emitting areas” (*T. latifolia* followed by *G. maxima* and *Spirogyra* sp.). Jacobs and Harrison (2014) investigate, in a microcosm experiment, the effects of *Lemna* sp. on N$_2$O emission not founding significant different fluxes between areas with and without floating vegetation with an average value of 0.012±0.009 µmol m$^{-2}$ h$^{-1}$. Inamori et al. (2008) reported significant N$_2$O different emissions among *T. latifolia*, *P. australis* and *Z. latifolia* with flux ranging from 0 to 52.8 mg m$^{-2}$ d$^{-1}$. The N$_2$O emission
peak was registered in *Z. latifolia* growth season (July–September) whereas the lower emissions were detected for *P. australis* in agreement with Inamori et al. (2007). No significant difference of N\textsubscript{2}O flux rate between vegetated and non-vegetated CWs was found during senescence season (Inamori et al., 2008). Chen et al. (2014), in a laboratory study using VSSF CW microcosms, investigated the effect of *T. latifolia* and its litter on density and abundance of three denitrifying genes (nirS, nirK and nosZ). Results showed that the presence of plants and litters had no significant direct impact on denitrifying genes, while they affected the denitrifying genes via alteration of dissolved oxygen and carbon sources. In fact denitrification is a bacterial process in which nitrogen oxides serve as terminal electron acceptors and these last are carried from an electron-donating substrate which is usually, but not exclusively, organic compounds (Vymazal, 2007).

Considering plant species Maltais-Landry et al. (2009a) monitored in a HSSSF CWs higher N\textsubscript{2}O emissions with *P. australis* than *T. angustifolia* and *P. arundinacea*. Wang et al. (2008a) compared three polyculture treatment beds planted with *P. australis*, *T. latifolia* and *Z. latifolia*, or *P. australis* and *T. latifolia*, or *P. australis* and *Z. latifolia* reporting that the presence of *Z. latifolia* stimulated the N\textsubscript{2}O emission probably due to the release of more organic matter and oxygen for ammonia-oxidizing bacteria (AOB) growth which enhanced biogeochemical activity in vegetation wetland ecosystems in growth seasons resulting in large fluxes on N\textsubscript{2}O. The authors reported that *Z. latifolia* root structure was favored by AOB for N\textsubscript{2}O formation and that in the growth season the N\textsubscript{2}O mean flux values were from 2 to 6-fold higher than those of the senescence period.

Chang et al. (2014) investigated the effect of plant species richness on N\textsubscript{2}O flux in a VSSF CWs microcosm vegetated with *Oenanthe hookeri* C. B. Clarke in Hook. f. Fl., *Phalaris arundinacea* Linn., *Reineckia carnea* (Andr.) Kunth, and *Rumex japonicus* Houtt using an artificial wastewater with NO\textsubscript{3} as the sole N source with a concentration of 336 mg L\textsuperscript{-1}. The authors, transplanting into microcosms 1, 2, 3 or 4 species in a complete block design, reported that N\textsubscript{2}O emission rate significantly increase with plant species richness in agreement with Sun et al. (2013) in hydroponic microcosms. Considering the experimental conditions results suggest that plant species richness enhance N\textsubscript{2}O emission rate under high nitrogen availability. Instead, Niklaus et al.,
(2006) reported that with increasing plant diversity, under nitrogen limited condition, the \( \text{N}_2\text{O} \) emission decrease.

Presence of vascular plants influences \( \text{N}_2\text{O} \) emission and tends to be species-specific (Ström et al., 2007), but regarding the effect of plants presence in the CW systems on \( \text{N}_2\text{O} \) emission, not unique literature data have been reported so far both lower (Wild et al., 2001; Johansson et al., 2003; Maltais-Landry et al., 2009b) and higher (Rückauf et al., 2004; Ström et al., 2007; Wang et al., 2008a; Inamori et al., 2008; Maltais-Landry et al., 2009b; García-Lledó et al., 2011) emission in planted CWs versus unplanted ones.

**CWs environmental balance**

Søvik et al. (2006) reported that the question then arises if CWs, used to protect freshwater ecosystems, are a solution to an environmental problem or if they substitute one problem with another by reducing water pollution, yet increasing GHGs emission. Pan et al. (2011), in estimated life-cycle GHGs emission study conclude that VSSF CW emitted only about the 50\% of \( \text{CO}_2(\text{eq}) \) respect the conventional system to remove 1 kg of BOD. Mander et al. (2008) calculating C balance in a HSSF CW found that it is a strong C sink, with an annual C sequestration of 1.5–2.2 kg C m\(^{-2}\) incorporated in phytomass and/or soil of wetland system. Mitsch et al. (2013) showed that most wetlands are net C sinks and not radiative sources of climate change adding that wetlands provide many ecosystem services in addition to C sequestration; considering also the savings that wetlands give us from fossil fuel consumption for the ecosystem services (e.g. water quality improvement) their service as carbon sinks is even greater. Maucieri et al. (2014b), at the end of five years study in a SFW CW with fluctuating hydroperiod treating agricultural drainage wastewater found a positive carbon balance with an annual soil C accumulation of 22.15 Mg ha\(^{-1}\) of equivalent \( \text{CO}_2 \) concluding that it can be considered a \( \text{CO}_2 \) sink.

Although the data reported in this review confirm the GHGs emission increase due to plants presence in CWs the \( \text{CO}_2(\text{eq}) \) balance needs to be considered in order to have a more complete view to answer the question posed by Søvik et al. (2006). In this case the \( \text{CO}_2(\text{eq}) \) plants biomass uptake balance GHGs emission showing a positive balance confirming that vegetation in CWs contributes to enhance the environmental value of this system of wastewater depuration.
Conclusions

Considering the CWs potential role as source of GHGs in this paper 127 articles (from 1980 to 2014) have been reviewed to investigate the roles of wastewater flow and composition, feeding strategy, environmental conditions and plant species used to vegetate CWs on the volumes and typology of GHGs emission.

- CWs typologies, substrate and feeding strategy influence directly and indirectly GHGs emission with higher CH\(_4\) emissions, under anoxic condition, and higher CO\(_2\) emissions under oxic condition. The N\(_2\)O emissions are not significantly influenced by CWs type.
- Wastewater characteristics influence GHGs emission mainly with their C/N ratio that should be 5:1 to obtain the lower GHGs emission.
- Intermittent CWs bed wastewater loading determine in the CWs beds an higher oxygen presence that decrease CH\(_4\) and increase CO\(_2\) and N\(_2\)O emissions.
- Environmental conditions greatly affects, directly and indirectly, the dynamics of GHGs emission from CWs mainly influencing plant metabolism and the heterotrophic microbial activities. Temperature is positively correlated with CO\(_2\), CH\(_4\) and N\(_2\)O emissions and solar radiation with CO\(_2\) and CH\(_4\) emissions.
- Several studies confirm the role of vascular plants in CWs on GHGs flux by their presence, phenology, density, and species composition. Plant species richness effect on GHGs emission has been investigate only for N\(_2\)O and CH\(_4\) and to reduce their emission monoculture have to prefer than polyculture. Therefore vegetation agronomic management can play an important influence to adjust GHGs emission from the CW systems.

Although plant presence on one hand increase CW GHG emissions on the other hand fix atmospheric carbon confirming that vegetation in CWs contributes to enhance the environmental value of this system of wastewater depuration.
### Appendix 1 – Greenhouse gases emission from un-vegetated CWs with their physical major characteristics, wastewater types and chemical composition (COD and/or BOD and/or TN).

| CW types | Substrate | Substrate depth (m) | Substrate depth (m) | Wastewater types | COD (mg L⁻¹) | BOD (mg L⁻¹) | TN (mg L⁻¹) | Wastewater depth (m) | Load (L m⁻² d⁻¹) | Retention time (d) | CO₂ (mg m⁻² h⁻¹) | CH₄ (mg m⁻² h⁻¹) | N₂O (mg m⁻² h⁻¹) | Reference |
|----------|-----------|---------------------|---------------------|------------------|-------------|-------------|------------|---------------------|-----------------|-----------------|----------------|----------------|----------------|----------------|----------|
| HSSF     | Coral Sand | 0.9                 |                     | Domestic wastewater | 53.4        |             |            | Between 12.6 and 75.8 | Between 18 and 3 | 80.21           | 3.57           | 1.40           |                | Sovik et al. 2006 |
| HSSF     | Inlet part: (0.1 m) large gravel (Ø 30-40 mm) beds: river gravel (Ø 10-15 mm) | 0.3                 | 1                   | Reconstituted effluent | 21.7        | 0.26        | 60         | 82.12               | 5.31            | 0.21            |                |                |                | Maltais Landry et al., (2009b)* |
| HSSF     | Inlet part: (0.1 m) large gravel (Ø 30-40 mm) beds: river gravel (Ø 10-15 mm) | 0.3                 | 1                   | Reconstituted fish farm effluent | 19.4        | 0.26        | 60         | 11.18               | 4.75            | 0.003           |                |                |                | Maltais Landry et al., (2009a)* |
| HSSF     | Sand      | 0.6                 | 2.5                 | Domestic wastewater | 0.45        |             |            | 60                 | 10.73           |                | 0.29           | 0.003          |                | Zhu et al. (2007) |
| HSSF     | Inlet part: (0.1 m) large gravel (Ø 30-40 mm) beds: river gravel (Ø 10-15 mm) | 0.3                 | 1                   | Reconstituted effluent | 21.7        | 0.26        | 60         | 79.57               | twrace          | 0.14            |                |                |                | Maltais Landry et al., (2009b)* |
| HSSF     | Inlet part: (0.1 m) large gravel (Ø 30-40 mm) beds: river gravel (Ø 10-15 mm) | 0.3                 | 1                   | Reconstituted fish farm effluent | 19.4        | 0.26        | 60         | 10.73               | 0.29            |                | 0.003          |                |                | Maltais Landry et al., (2009a)* |
| FWS      | Sand      | 0.3                 | 2.5                 | Domestic wastewater | 0.45        |             |            | 60                 | 0.15            |                | 0.37           |                |                | Zhu et al. (2007) |
| FWS      | Soil      | 900                 |                     | Municipal wastewater | 8           | 0.1-0.3     | 64.3       | 7.6                 | 245             |                | 245            | 0.248          |                | Johansson et al. (2003) and Johansson et al. (2004) |
| VSSF     | Coral Sand | 0.05                |                     | Domestic wastewater | 52.6        |             |            | Between 12.6 and 75.8 | Between 18 and 3 | 1978.47        | 7.22           | 31.44          |                | Sovik et al. 2006 |
| VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 18 cm gravel | 0.61                | 0.25                | Artificial domestic | 50          | 15          | 42         | 7                  | 0.15           |                |                |                |                | Inamori et al. (2008) |
| VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 18 cm gravel | 0.61                | 0.25                | Artificial domestic | 100         | 30          | 42         | 7                  | 0.16           |                |                |                |                | Inamori et al. (2008) |
| VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 6 cm coarse sand, 12 cm gravel | 0.61                | 0.25                | Artificial domestic | 50          | 15          | 0.67       | 17                 | 7              |                | 0.1375         |                |                | Wang et al. (2008a) |
**Appendix 1 (continued)**

| CW types | Substrate | Substrate depth (m) | Surface (m²) | Wastewater types | COD (mg L⁻¹) | BOD (mg L⁻¹) | TN (mg L⁻¹) | Wastewater depth (m) | Load (L m⁻² d⁻¹) | Retention time (d) | CO₂ (mg m⁻² h⁻¹) | CH₄ (mg m⁻² h⁻¹) | N₂O (mg m⁻² h⁻¹) | Reference |
|----------|-----------|---------------------|--------------|------------------|--------------|--------------|-------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------|
| VSSF     | From upper to lower layers: 43 cm sand (Ø <1mm) 18 cm gravel | 0.61 | 0.25 | Artificial domestic | 50 | 15 | 0.67 | 0.42 | 7 | 11.13 | | | | Wang et al., (2008b) |
| VSSF     | From upper to lower layers: 43 cm sand (Ø <1mm) 18 cm gravel | 0.61 | 0.25 | Artificial domestic | 100 | 30 | 0.67 | 0.42 | 7 | 27.26 | | | | | Wang et al., (2008b) |
| VSSF (eco-filter) | 35 cm artificial soil (99:1 peat soil:wood chips) 5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm) | 0.53 | 0.16 | Sodium dodecyl sulfate | 101.57 | 43.89 | 262.5 | | | | | | | | | Huang et al., (2014) |
| VSSF (eco-filter) | From upper to lower layers: 35 cm artificial soil (99:1 peat soil:wood chips) 5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm) | 0.53 | 0.16 | Sodium dodecyl sulfate | 202.75 | 44.33 | 262.5 | | | | 370 | 11.65 | | | | | Huang et al., (2014) |
| VSSF (eco-filter) | 35 cm artificial soil (99:1 peat soil:wood chips) 5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm) | 0.53 | 0.16 | Sodium dodecyl sulfate | 406.93 | 42.54 | 262.5 | | | | 367.77 | 15.87 | | | | | Huang et al., (2014) |

*greenhouse gases emission data taken from graph; FWS = free water surface CW; VSSF = vertical subsurface flow CW; HSSF = horizontal subsurface flow CW
### Appendix 2 – Greenhouse gases emission from CWs vegetated by monoculture, CWs physical major characteristics, wastewater types and chemical composition (COD and/or BOD and/or TN).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>CW types</th>
<th>Substrate</th>
<th>Substrate depth (m)</th>
<th>Surface (m²)</th>
<th>Wastewater types</th>
<th>COD (mg L⁻¹)</th>
<th>BOD (mg L⁻¹)</th>
<th>TN (mg L⁻¹)</th>
<th>Wastewater depth (m)</th>
<th>Load (L m⁻² d⁻¹)</th>
<th>Retention time (d)</th>
<th>CO₂ (mg m⁻² h⁻¹)</th>
<th>CH₄ (mg m⁻² h⁻¹)</th>
<th>N₂O (mg m⁻² h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. calamus</td>
<td>VSSF</td>
<td>From upper to lower layer: 25 cm slag (Ø 1.5 cm), 20 cm gravel (Ø 1.2 cm)</td>
<td>0.45</td>
<td>0.6</td>
<td>Syntetic wastewater</td>
<td>102.32</td>
<td>40.14</td>
<td>66.7</td>
<td>1.5</td>
<td>283.57</td>
<td>1.36</td>
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<td>Yan et al. (2012)</td>
</tr>
<tr>
<td>A. calamus</td>
<td>VSSF</td>
<td>From upper to lower layer: 25 cm slag (Ø 1.5 cm), 20 cm gravel (Ø 1.2 cm)</td>
<td>0.45</td>
<td>0.6</td>
<td>Syntetic wastewater</td>
<td>202.64</td>
<td>81.06</td>
<td>66.7</td>
<td>1.5</td>
<td>396.59</td>
<td>1.97</td>
<td></td>
<td></td>
<td></td>
<td>Yan et al. (2012)</td>
</tr>
<tr>
<td>A. calamus</td>
<td>VSSF</td>
<td>From upper to lower layer: 25 cm slag (Ø 1.5 cm), 20 cm gravel (Ø 1.2 cm)</td>
<td>0.45</td>
<td>0.6</td>
<td>Syntetic wastewater</td>
<td>203.56</td>
<td>22.06</td>
<td>66.7</td>
<td>1.5</td>
<td>466.97</td>
<td>2.86</td>
<td></td>
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</tr>
<tr>
<td>A. calamus</td>
<td>VSSF</td>
<td>From upper to lower layer: 25 cm slag (Ø 1.5 cm), 20 cm gravel (Ø 1.2 cm)</td>
<td>0.45</td>
<td>0.6</td>
<td>Syntetic wastewater</td>
<td>204.71</td>
<td>41.56</td>
<td>66.7</td>
<td>1.5</td>
<td>419.19</td>
<td>2.02</td>
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<tr>
<td>A. calamus</td>
<td>VSSF</td>
<td>From upper to lower layer: 25 cm slag (Ø 1.5 cm), 20 cm gravel (Ø 1.2 cm)</td>
<td>0.45</td>
<td>0.6</td>
<td>Syntetic wastewater</td>
<td>404.33</td>
<td>41.26</td>
<td>66.7</td>
<td>1.5</td>
<td>457.34</td>
<td>2.34</td>
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<td>Yan et al. (2012)</td>
</tr>
<tr>
<td>A. calamus</td>
<td>VSSF (eco-filter)</td>
<td>5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm)</td>
<td>0.53</td>
<td>0.16</td>
<td>Sodium dodecyl sulfate</td>
<td>101.57</td>
<td>43.89</td>
<td>262.5</td>
<td>14.64</td>
<td>428.6</td>
<td></td>
<td></td>
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<td>Huang et al., (2014)</td>
</tr>
<tr>
<td>A. calamus</td>
<td>VSSF (eco-filter)</td>
<td>5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm)</td>
<td>0.53</td>
<td>0.16</td>
<td>Sodium dodecyl sulfate</td>
<td>202.75</td>
<td>44.33</td>
<td>262.5</td>
<td>19.32</td>
<td>632.44</td>
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<td>Huang et al., (2014)</td>
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</table>
### Appendix 2 (Continued)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>CW types</th>
<th>Substrate</th>
<th>Substrate depth (m)</th>
<th>Surface (m²)</th>
<th>Wastewater types</th>
<th>COD (mg L⁻¹)</th>
<th>BOD (mg L⁻¹)</th>
<th>TN (mg L⁻¹)</th>
<th>Wastewater depth (m)</th>
<th>Load (L m² d⁻¹)</th>
<th>Retention time (d)</th>
<th>CO₂ (mg m² h⁻¹)</th>
<th>CH₄ (mg m² h⁻¹)</th>
<th>N₂O (mg m² h⁻¹)</th>
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</thead>
<tbody>
<tr>
<td><em>A. calamus</em></td>
<td>VSSF</td>
<td>From upper to lower layers: 35 cm artificial soil (99:1 peat soil:wood chips) 5 cm mixture of sand and gravel (Ø 1-5 mm) 10 cm ceramsite (Ø 20-40 mm) 3 cm large gravel (Ø 35-45 mm)</td>
<td>0.53</td>
<td>0.16</td>
<td>Sodium dodecyl sulfate</td>
<td>406.93</td>
<td>42.54</td>
<td>262.5</td>
<td>618.57</td>
<td>14.38</td>
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<td></td>
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<tr>
<td><em>Carex sp.</em></td>
<td>OGF</td>
<td>Peat</td>
<td>8200</td>
<td></td>
<td>Municipal wastewater</td>
<td>59.7</td>
<td>35.2</td>
<td>1.9</td>
<td>351.39</td>
<td>6.91</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td>Sovik et al. 2006</td>
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<tr>
<td><em>G. maxima</em></td>
<td>FWS</td>
<td>Soil</td>
<td>900</td>
<td></td>
<td>Municipal wastewater</td>
<td>8</td>
<td>0.1-0.3</td>
<td>64.3</td>
<td>160</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Johansson et al. (2003) and Johansson et al. (2004)</td>
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<tr>
<td><em>L. minor</em></td>
<td>FWS</td>
<td>Soil</td>
<td>900</td>
<td></td>
<td>Municipal wastewater</td>
<td>8</td>
<td>0.1-0.3</td>
<td>64.3</td>
<td>675</td>
<td>0.094</td>
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<td><em>P. arundinacea</em></td>
<td>FWS</td>
<td>Soil</td>
<td>1000</td>
<td></td>
<td>Municipal wastewater</td>
<td>8</td>
<td>0.1-0.3</td>
<td>64.3</td>
<td>318</td>
<td>0.248</td>
<td></td>
<td></td>
<td></td>
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<td>Liu et al. (2009)*</td>
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<tr>
<td><em>P. arundinacea</em></td>
<td>HSSF</td>
<td>Inlet part: (0.1 m) large gravel (Ø 30-40 mm), beds: river gravel (Ø 10-15 mm)</td>
<td>0.3</td>
<td>0.1</td>
<td>Reconstituted fish farm effluent</td>
<td>19.4</td>
<td>60</td>
<td>31.3</td>
<td>2.25</td>
<td>0.001</td>
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<td></td>
<td></td>
<td></td>
<td>Maltais Landry et al., (2009a)*</td>
</tr>
<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>From upper to lower layer: 35 cm washed sand, 5 cm gravel (Ø 40-50 mm)</td>
<td>0.4</td>
<td>0.18</td>
<td>Synthetic wastewater</td>
<td>362</td>
<td>50</td>
<td>0.5</td>
<td>11.9</td>
<td>7 (4 d flooding, 3 d drying)</td>
<td>from 0.16 to 0.31</td>
<td>Jia et al. (2011)</td>
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<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>From upper to lower layer: 35 cm washed sand, 5 cm gravel (Ø 40-50 mm)</td>
<td>0.4</td>
<td>0.18</td>
<td>Synthetic wastewater</td>
<td>362</td>
<td>50</td>
<td>0.5</td>
<td>11.9</td>
<td>7 (7 d flooding)</td>
<td>from 0.17 to 0.32</td>
<td>Jia et al. (2011)</td>
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<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>From upper to lower layer: 42 cm fine sand (Ø 1-2 mm), 6 cm coarse sand (Ø 3-5 mm), 12 cm gravel (Ø 10-20 mm)</td>
<td>0.6</td>
<td>12</td>
<td>Domestic wastewater</td>
<td>140</td>
<td>200</td>
<td>100</td>
<td>0.7-0.75</td>
<td>41.7</td>
<td>6</td>
<td>26</td>
<td>0.1</td>
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<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>Sediment</td>
<td>44000</td>
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<td>Municipal wastewater</td>
<td>66.1</td>
<td>82.4</td>
<td>1.5</td>
<td>397.22</td>
<td>13.16</td>
<td>0.01</td>
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<td>Sovik et al. 2006</td>
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### Appendix 2 (Continued)

<table>
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<tr>
<th>Plant species</th>
<th>CW types</th>
<th>Substrate</th>
<th>Substrate depth (m)</th>
<th>Surface ( (m^2) )</th>
<th>Wastewater types</th>
<th>COD (mg L(^{-1}))</th>
<th>BOD (mg L(^{-1}))</th>
<th>TN (mg L(^{-1}))</th>
<th>Wastewater depth (m)</th>
<th>Load (L m(^{-2}) d(^{-1}))</th>
<th>Retention time (d)</th>
<th>( CO_2 ) (mg m(^{-2}) h(^{-1}))</th>
<th>( CH_4 ) (mg m(^{-2}) h(^{-1}))</th>
<th>( N_2O ) (mg m(^{-2}) h(^{-1}))</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>Soil</td>
<td></td>
<td>50000</td>
<td>Stream water</td>
<td>about 7</td>
<td>2</td>
<td>from 0.03 to 1.35</td>
<td>0.49</td>
<td>0.73</td>
<td>0.11</td>
<td>0.10</td>
<td>1.03</td>
<td>de Klain and van der Werf (2014)</td>
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<td>FWS</td>
<td>Washed sand (Ø 1-2 mm)</td>
<td>0.35</td>
<td>0.18</td>
<td>Synthetic wastewater</td>
<td>4.71</td>
<td>51.99</td>
<td>0.45</td>
<td>7</td>
<td>Wu et al. (2009)*</td>
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<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>Washed sand (Ø 1-2 mm)</td>
<td>0.35</td>
<td>0.18</td>
<td>Synthetic wastewater</td>
<td>123.11</td>
<td>55.14</td>
<td>0.45</td>
<td>7</td>
<td>Wu et al. (2009)*</td>
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<tr>
<td><em>P. australis</em></td>
<td>FWS</td>
<td>Washed sand (Ø 1-2 mm)</td>
<td>0.35</td>
<td>0.18</td>
<td>Synthetic wastewater</td>
<td>305.65</td>
<td>54.33</td>
<td>0.45</td>
<td>7</td>
<td>Wu et al. (2009)*</td>
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<td>BOD (mg L⁻¹)</td>
<td>TN (mg L⁻¹)</td>
<td>Wastewater depth (m)</td>
<td>Load (L m⁻² d⁻¹)</td>
<td>Retention time (d)</td>
<td>CO₂ (mg m⁻² h⁻¹)</td>
<td>CH₄ (mg m⁻² h⁻¹)</td>
<td>N₂O (mg m⁻² h⁻¹)</td>
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<td>VSSF</td>
<td>From upper to lower layer: 43 cm sand (Ø &lt; 1 mm), 18 cm gravel</td>
<td>0.61</td>
<td>0.25</td>
<td>Artificial domestic wastewater</td>
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<td>From upper to lower layer: 43 cm sand (Ø &lt; 1 mm), 18 cm gravel</td>
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<td>0.25</td>
<td>Artificial domestic wastewater</td>
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<td>67.53</td>
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<td>Wang et al., (2008b)</td>
</tr>
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<td><em>Z. latifolia</em></td>
<td>VSSF</td>
<td>From upper to lower layer: 43 cm sand (Ø &lt; 1 mm), 18 cm gravel</td>
<td>0.61</td>
<td>0.25</td>
<td>Artificial domestic wastewater</td>
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<td></td>
<td>0.42</td>
<td>7</td>
<td>270.28</td>
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</table>

* greenhouse gases emission data taken from graph; FWS = free water surface CW; VSSF = vertical subsurface flow CW; HSSF = horizontal subsurface flow CW; OGF = overland and groundwater flow wetlands
### Appendix 3 – Greenhouse gases emission from CWs vegetated by polyculture, CWs physical major characteristics, wastewater types and chemical composition (COD and/or BOD and/or TN).

| Plant species | CW types | Substrate                  | Substrate depth (m) | Surface (m²) | Wastewater types          | COD (mg L⁻¹) | BOD (mg L⁻¹) | TN (mg L⁻¹) | Wastewater depth (m) | Load (L m⁻² d⁻¹) | Retention time (d) | CO₂ (mg m⁻² h⁻¹) | CH₄ (mg m⁻² h⁻¹) | N₂O (mg m⁻² h⁻¹) | Reference |
|---------------|----------|----------------------------|---------------------|--------------|--------------------------|--------------|--------------|--------------|----------------------|------------------|------------------|------------------|------------------|------------------|---------------------|-----------|
| *I. pseudocorus*, *P. australis* | HSSF     | Sand                       | 156.3               | 1034         | Hospital wastewater      | 96.5         | 7-7.4        | 6-8          | 8.17                  | 0.23             |                  |                  |                  |                  | Mander et al. (2005); Teiter and Mander (2005); Sovik et al. (2006) |
| *T. latifolia, S. sylvaticus*     | HSSF     | Sand                       | 156.3               | 1034         | Hospital wastewater      | 96.5         | 7-7.4        | 6-8          | 0.46                  | 0.23             |                  |                  |                  |                  | Mander et al. (2005); Teiter and Mander (2005); Sovik et al. (2006) |
| *T. latifolia, P. australis*      | HSSF     | Crushed limestone (Ø 15-20 mm) | 365                | 977          | Raw municipal wastewater | 43.1         | 12.2-14.2    | 3-4          | 6.04                  | 0.19             |                  |                  |                  |                  | Mander et al. (2005); Teiter and Mander (2005); Sovik et al. (2006) |
| *P. australis*, *T. latifolia, Z. latifolia* | VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 6 cm coarse sand, 12 cm gravel | 0.61          | 0.25         | Artificial domestic wastewater | 50.15 | 0.67 | 17 | 7 | 0.408333 |                  |                  |                  |                  | Wang et al. (2008a) |
|                               | VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 6 cm coarse sand, 12 cm gravel | 0.61          | 0.25         | Artificial domestic wastewater | 50.15 | 0.67 | 17 | 7 | 0.141667 |                  |                  |                  |                  | Wang et al. (2008a) |
| *P. australis*, *T. latifolia, Z. latifolia* | VSSF     | From upper to lower layer: 43 cm sand (Ø < 1 mm), 6 cm coarse sand, 12 cm gravel | 0.61          | 0.25         | Artificial domestic wastewater | 50.15 | 0.67 | 17 | 7 | 0.2625 |                  |                  |                  |                  | Wang et al. (2008a) |
| *T. latifolia, T. angustifolia*   | FWS      | Peat                       | 22000               | 1705         | Agriculture drainage water | 20.3         | 0.4          | 18           | 0.2                  | 0.02             |                  |                  |                  |                  | Wild et al., (2001) |
| *T. latifolia, T. angustifolia*   | FWS      | Peat                       | 26000               | 1705         | Agriculture drainage water | 10.2         | 0.2          | 18           | 1.18                 | -0.007           |                  |                  |                  |                  | Wild et al., (2001) |
## Appendix 3 (continued)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>CW types</th>
<th>Substrate</th>
<th>Substrate depth (m)</th>
<th>Surface (m²)</th>
<th>Wastewater types</th>
<th>COD (mg L⁻¹)</th>
<th>BOD (mg L⁻¹)</th>
<th>TN (mg L⁻¹)</th>
<th>Wastewater depth (m)</th>
<th>Load (L m⁻² d⁻¹)</th>
<th>Retention time (d)</th>
<th>CO₂ (mg m⁻² h⁻¹)</th>
<th>CH₄ (mg m⁻² h⁻¹)</th>
<th>N₂O (mg m⁻² h⁻¹)</th>
<th>Reference</th>
</tr>
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<tr>
<td><em>Menyanthes trifoliata,</em> <em>Carex lasio carpa,</em> <em>Potentilla palustris</em> <em>Sphagnum angustifolium,</em> <em>S. papillosum,</em> <em>Menyanthes trifoliata</em></td>
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<td></td>
<td>OGF</td>
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<td>21.6</td>
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<td>5.83</td>
<td>0.01</td>
<td>Liikanen et al. (2006)</td>
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</tbody>
</table>

FWS = free water surface CW; VSSF = vertical subsurface flow CW; HSSF = horizontal subsurface flow CW; OGF = overland and groundwater flow wetlands
Chapter II
Carbon dioxide emissions from horizontal sub-surface constructed wetlands in the Mediterranean Basin.
Abstract

Constructed wetlands (CWs) are widely used natural-like systems for wastewater treatment where organic matter is removed through carbon dioxide (CO₂) emissions. Several studies have been conducted regarding emissions and the sequestration of CO₂ in CWs in the Northern Hemisphere; however, to the best of our knowledge, no studies have been performed in the Mediterranean Basin. This work quantified daily and cumulative CO₂ emissions from a full-scale CW horizontal subsurface flow (HSSF) bed during semiarid Mediterranean spring climate conditions. The average daily CO₂–C that was released in the atmosphere during the first 50 days ranged from approximately 17.5% to 32.6% of the C that was removed from wastewater. Considering both the Phragmites australis aerial part dry matter production (0.83 kg m⁻²) and the average CO₂–C emissions, after 50 days of vegetative regrowth, the HSSF bed was demonstrated to act as a CO₂ sink. The cumulative CO₂ efflux was 452.15 ± 50.40 CO₂ g m⁻² and 276.02 ± 12.07 CO₂ g m⁻² for vegetated and unvegetated sites, respectively.

Introduction

Constructed wetlands (CWs) are widely used to treat different wastewaters (Vymazal, 2009), where mineral and organic pollutants are removed through various physical and biochemical mechanisms. In such natural-like systems, organic matter is further removed through carbon dioxide (CO₂) evolution. Considering that the ratio of CO₂–C that is emitted by bed respiration to carbon (C) that is removed from wastewater alone is often >100% during the growing season, this ratio suggests that the C that is lost to the atmosphere as CO₂ exceeds the C that is removed from wastewater (VanderZaag et al., 2010). This result is most likely due to plant root exudates that increase the C input into the bed system by supporting various heterotrophic microbial processes (Picek et al., 2007).

Several studies have considered CO₂ emissions and sequestration, as well as CH₄ emissions, in natural wetlands (Brix et al., 2001; Heinsch et al., 2004; Mitch et al., 2013) and CWs (Picek et al., 2007; Mander et al., 2008) in relation to meteorological (Liikanen and et al., 2006) and hydrological conditions (Altorl and Mitsch, 2008) because wetlands can be a C source or sink (Heikkinen et al., 2002; Ström et al., 2007),
depending on their age (Zemanová et al., 2010) and operation, as well as environmental conditions, such as location and climate (Scholz, 2011). These studies have been performed in CWs in continental areas of the Northern Hemisphere, but not in the Mediterranean Basin.

The aim of this work was to quantify daily and cumulative CO$_2$ emission from a horizontal subsurface flow (HSSF) CW in semiarid Mediterranean spring climate conditions (Sicily, Italy), comparing vegetated and unvegetated areas.

**Materials and Methods**

**Study site**

This research was conducted in a full-scale CW treatment plant in San Michele di Ganzaria (Eastern Sicily, 37°16’ N, 14°25’ E), which is a rural community of approximately 5,000 inhabitants. This area is characterized by a Mediterranean semi-arid climate, with a mean annual rainfall of 600 mm and with a mean daily temperature of 18 °C (average data 2008-2011). The CW treatment system consists of an HSSF bed with a surface area of approximately 2080 m$^2$ (33 m $\times$ 63 m) and with a design flow rate of approximately 455 m$^3$ d$^{-1}$, which has been in operation since 2006 (Barbagallo et al., 2011). The CW is used for the tertiary treatment of the urban effluent (approximately 4 L s$^{-1}$) from a conventional wastewater treatment plant (trickling filter). The end section of the HSSF bed functions as a free water surface, with an area of approximately 100 m$^2$. The filter bed, which is filled with 8–10 mm volcanic gravel (0.40 porosity), is 0.6 m deep on average and has been planted with *Phragmites australis* (Cav.) Trin. ex Steud at a density of four rhizomes m$^{-2}$. Vegetation cutting were scheduled every two years; the last vegetation harvesting was performed in winter 2010 by cutting the stems at a height of 10 cm above the substrate surface. The water table in the bed was constant throughout the study, at approximately 18 cm from the surface, and the bed slope is approximately 1%.

**Environmental variables**

The following climatic data were recorded by a weather station that was close to the experimental site: rain (mm), air temperature (°C), wind speed (m s$^{-1}$), relative humidity
(%), and solar radiation (MJ m$^{-2}$ d$^{-1}$). Evapotranspiration was calculated with the Penman-Monteith model, which used Kc that was previously determined for the experimental site (Milani and Toscano, 2013).

**Water sampling and analysis**

The following water physicochemical parameters were evaluated at the inlet and outlet of the HSSF according to APHA (1998) methods: total suspended solids (TSS) at 105 °C, COD, NH$_4^+$-H, NO$_2^-$-N, NO$_3^-$-N, total nitrogen (TN) and orthophosphates (PO$_4^{3-}$-P). The evaluation of treatment performance, which was based on the mass pollutant removal efficiency percentage, was calculated using average inflow and outflow pollutant concentrations, considering the sum of wastewater inlet and precipitation as the inlet volume and the inlet volume minus the evapotranspiration as the outflow volume.

**CO$_2$ emission**

The monitoring was performed from April 10$^{th}$ (vegetative regrowth) to July 18$^{th}$ 2012 for 100 days. In the first 50 days, daily CO$_2$ emissions were measured in 3 areas of the HSSF bed, which was vegetated with *P. australis* (6.5 m (P1), 31.5 m (P2) and 56.5 m (P3) from wastewater inflow), and in one unvegetated area of the HSSF bed (6.5 m from inflow (P4)) (Fig. 1) with different water column lengths throughout the study site (0.55 m in sites P1 and P4, 0.75 m in site P2 and 0.95 m in site P3). In the following 50 days, measurements were only taken in P2 and P4 to calculate cumulative CO$_2$ emissions. The CO$_2$ bed respiration included gas emissions due to microbial metabolism and root respiration; aboveground plant photosynthetic tissue respiration, in agreement with Mander et al. (2008), was not considered because the proportion of C that was respired by the shoot was first assimilated by plant gross photosynthesis, with an annual difference that was equal to zero.
Figur 1 – HSSF bed sampling points.

$CO_2$ emissions were estimated in situ using the static-stationary chamber technique. The cylindrical dark chambers, which were composed of PVC with an effective volume of approximately 3 L, were 35 cm in height and 16 cm wide. The bottom part (20 cm) was permanently inserted in the gravel substrate and the chamber was sealed with a lid. In the chamber, $CO_2$ that was emitted from the bed was continuously trapped in a sodium hydroxide (NaOH) solution trap (Knoepp and Vose, 2002). NaOH base traps were chosen because these traps have been successfully used in a previous field study (Welker et al., 2004), where long-term remote sampling of $CO_2$ efflux was required. However, the $CO_2$ rates from this technique are typically conservative because of the reliance on diffusion. To reduce the experimental error of this technique to acceptable levels, we have made the following enhancements: 1) the trap solution was replaced every five days to avoid any daily influence on the $CO_2$ flux estimate and to reduce the $CO_2$ atmospheric influence when the static chamber was open to replace the trap; 2) with this frequency, the NaOH solution was maintained under 70% of its $CO_2$ absorption capacity, in agreement with Sharkov (1984), who reported no change in the absorption rate when the alkali was used until 70%; 3) to accelerate $CO_2$ absorption, a high jar/chamber surface ratio (37.5%) was used that was greater than values that were reported by Jensen et al. (1996), which have been used for Danish and New Zealand experimental sites jar traps with a surface of 16% and 26% of the chamber area, respectively. Moreover, the high jar/chamber surface ratio reduced the underestimated $CO_2$ measures at high soil respiration rates (above 300 mg m$^{-2}$ h$^{-1}$), which may artificially decrease the microorganism respiratory activity that reduces the measured
CO₂ fluxes (Yim et al., 2002). In each sampling area, the chambers were installed in two microsites to replicate the measurements. During the first 50 days, the ratio of CO₂–C emitted in the atmosphere, with respect to the C that was removed from wastewater by the CW system, was also considered, taking into account the organic load. The C input by wastewater in the HSSF bed was calculated using the average inflow and outflow water volumes and C concentration. Carbon concentrations in the inflow and outflow waters were calculated using COD that was converted to C using the multiplicative coefficients of 0.31 and 0.36, respectively (Pitter, 1999).

**Plant biomass**

Six samples of five P. australis plants each (thirty plants total) were collected from the three vegetated sites (P1, P2, and P3). Samples were harvested at the end of the first monitoring period at the 13th – 14th leaf. Biomass dry weight was determined by drying in a thermo-ventilated oven at 65 °C until the constant weight was reached. To calculate the CO₂–C fixed in the aboveground biomass from the atmosphere, C dry matter content was determined using a CNS Macrovario combustion analyzer (Elementar Analysensysteme GmbH, Germany).

**Statistical analysis**

The normality of data was checked using the Kolmogorov–Smirnov, Lilliefors' and Shapiro–Wilk's tests. CO₂ emissions from the study site did not show a normal distribution, therefore non-parametric tests, Kruskal–Wallis and Mann–Whitney, were used to check the significance of differences.

**Results and Discussion**

**Environmental variables**

Climatic data that were recorded during the monitoring period (April-July 2012) (Fig. 2) are typical for Mediterranean spring-summer seasons. In fact, during the study period, the cumulative rainfall was 72 mm, and the average daily air temperature reached 19.8 °C, with its maximum value on July 13th (43.4 °C). In contrast, during the same days, the average solar radiation that reached the canopy was 27.4 MJ m⁻² d⁻¹, with the highest
monthly average intensity of 30 MJ m\(^{-2}\) in June. The average wind speed was generally below 1 m s\(^{-1}\).

![Figure 2](image)

**Figure 2** – Temperature, rainfall, solar radiation and wind speed recorded during study period.

The average daily evapotranspiration during the study period was 18.0 mm, with the highest value in the second half of July (48.7 mm) and the lowest value in the second half of April (4.9 mm). The multiple linear regression model that compared the relation
between CO₂ emissions and the environmental variables: rain, Tₘₐₓ, Tₘᵋᵣₐᵋᵣₑ and Tₐᵥₑᵋᵣₐᵋᵥₑ did not show any regression.

**Water analysis**

The wastewater quality in the influent and effluent of the HSSF from April-July 2012 are reported in table 1. The effluent quality was excellent, and the COD and TSS values, in particular, were always below the Italian law discharge parameters (35 and 125 mg L⁻¹, respectively), which confirmed the high performance that was previously reported by Barbagallo et al. (2011).

**Table 1** – Wastewater quality in the influent (IN) and effluent (OUT) of the HSSF in the study period.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>04 April 2012</th>
<th>07 May 2012</th>
<th>19 June 2012</th>
<th>25 July 2012</th>
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<td>7.13</td>
<td>7.54</td>
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<td>TSS (mg L⁻¹)</td>
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<td>0.001</td>
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<td>NO₃⁻-N (mg L⁻¹)</td>
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</table>

**CO₂ emissions**

Carbon dioxide emissions from the three vegetated sampling areas of the HSSF bed showed different values during the monitoring period. The static chambers that were positioned in the middle of the bed (site P2) recorded higher significant CO₂ flow than site P1 (Fig. 3) with an average five-day cumulative emission of 21.2 g m⁻² (P2) and 11.4 g m⁻² (P1). Site P3 showed a significantly higher emission (17.4 g m⁻²) than site P1. The lower emission that was detected in the initial part of the bed (P1) compared with the other vegetated sites (P2 and P3) is undoubtedly interesting, although this result should be interpreted with caution because our experiment only monitored 50 days. Nevertheless, this result could be due to the different heights of wastewater columns in the vegetated
sites, which influence the development of plant root systems, which determine different microbial respiration.

**Figure 3** – Box-plot diagram of carbon dioxide daily emissions from different measure sites. Different letters indicate significant differences at $p < 0.05$ by Kruskal–Wallis test.

The average daily CO$_2$ efflux of vegetated areas varied from $2.05 \pm 0.58$ g m$^{-2}$ d$^{-1}$ (site P1 on May 20$^{th}$) to $5.14 \pm 0.77$ g m$^{-2}$ d$^{-1}$ (site P2 on April 10$^{th}$). In the unvegetated area, the
average daily CO₂ emissions ranged from 1.26 ± 1.09 g m⁻² d⁻¹ on April 10th to 3.53 ± 0.43 g m⁻² d⁻¹ on April 20th. CO₂ emissions from vegetated sites were significantly higher than the unvegetated one (Fig. 4), in agreement with Strom et al. (2007), who reported a significantly higher emission from vegetated than unvegetated areas, which indicated that the presence of vegetation was of great importance for total ecosystem respiration. Maltais-Landry et al. (2009b) reported the significant effect of the presence of macrophytes on CO₂-C emissions, with higher fluxes in planted units compared with unplanted ones. Higher emissions in the *P. australis* planted area were expected because this macrophyte sustains a larger heterotrophic bacterial population with its air-root system release. In this species, air enters through the shoots and passes by the aerenchyma down to the rhizomes (Armstrong and Armstrong, 1991), with an higher flow rate than other macrophytes that are commonly used in CWs (5.29 ± 0.40 cm³ min⁻¹ culm⁻¹; Brix et al., 1992). To our knowledge, no study has been conducted in the Mediterranean climate areas on HSSF CW bed respiration. Instead, several studies were conducted in higher latitudes. In southern Sweden, Ström et al. (2007) reported from a peat-based CW site, which was vegetated with *P. australis*, an average CO₂ flux of 25.10 ± 4.74 g m⁻² d⁻¹, which was measured from April to May. Søvik et al. (2006), in a North Europe comparative study on GHG emissions from CWs, have reported a summer average CO₂ emission of 12.10 ± 2.38 g m⁻² d⁻¹ from a Polish common reed HSSF bed. The average daily ratio of CO₂-C that was released in the atmosphere to the C that was removed from wastewater in the first 50 days ranged from approximately 17.5% (site P1) to 32.6% (site P2). Regarding the vegetated and unvegetated sites (P1 and P4), no significant differences were measured in the CO₂-C flux that was emitted in the atmosphere (Fig. 3a), with the lowest values among the other sites. This contrasting result could be explained considering that both sites were close to the wastewater inflow point (6.5 m), where there was a significant accumulation of sludge (data not shown), which can be considered an anoxic rhizosphere condition, irrespective of common reed root air release. The total CO₂ emissions at the end of the 100 days were 452.15 ± 50.40 g m⁻² and 276.02 ± 12.07 g m⁻² for vegetated (P2) and unvegetated (P4) sites, respectively. During the study period, the cumulative CO₂ emissions from the vegetated and unvegetated sites have
shown a difference, except during the first 15 days of monitoring when plant growth was negligible after the winter dormant months, which confirmed the role of plants in bacterial metabolism and CO$_2$ emissions, as illustrated by the seasonal variation (Liikanen et al., 2006). Therefore, higher CO$_2$ fluxes from the vegetated site may be a function of intensified bacterial activity (Gagnon et al., 2007) because of more labile C that is accessible via plant exudates (Zemanovà et al., 2010), which represented up to 20% of aboveground biomass production (Picek et al., 2007). The main contribution of plants to CO$_2$ emission can be determined by observing the last 15 days, when the cumulative CO$_2$ emissions showed a negligible increase in the unvegetated site compared with the vegetated ones. Moreover, the wastewater COD value was the lowest that was measured (35.71 mg L$^{-1}$). As known, aerobic microbial reactions, which mineralize more organic carbon than anaerobic reactions (Zemanovà et al., 2010), are improved through root oxygen and C exudates in CWs (Tanner, 2001; Vymazal, 2011).

**Aboveground biomass**

The aboveground dry matter production at the end of the first monitoring period, which was an average of the three sampling points, was 0.83 kg m$^{-2}$, with 42.8% of C content fixed in the shoots, which was equal to 0.35 kg m$^{-2}$. Considering the average CO$_2$–C emission from the HSSF bed during the same period, the carbon that was released into the atmosphere was 0.23 kg m$^{-2}$. The belowground biomass variation was not considered because the root system had already reached its steady turnover, with the bed in operation since 2006.

**Conclusions**

The cumulative CO$_2$ effluxes were significantly higher in vegetated than unvegetated areas; the vegetated areas exhibited different emissions with higher flows in the middle part of the bed. Although this experiment was performed for only several months and that a full C balance was not calculated, the data regarding CO$_2$–C emission and plant aboveground biomass production demonstrated that the HSSF bed for municipal wastewater treatment in the Sicilian Mediterranean spring climate conditions acts as a CO$_2$ sink. Further long-term investigations are required to improve datasets regarding the CO$_2$ balance in Mediterranean HSSF CWs.
Chapter III
Comparison of carbon balance in Mediterranean pilot constructed wetlands vegetated with different C4 plant species
Abstract

This study investigates CO₂ and CH₄ emissions and C budgets in a horizontal subsurface flow pilot-plant constructed wetland (CW) with beds vegetated with *Cyperus papyrus* L., *Chrysopogon zizanioides* (L.) Roberty and *Mischantus x giganteus* Greef et Deu. in the Mediterranean basin (Sicily) during the first year plants growing season. At the end of the vegetative season *M. giganteus* showed the higher biomass accumulation (7.4 kg m⁻²) followed by *C. zizanioides* (5.3 kg m⁻²) and *C. papyrus* (1.8 kg m⁻²). Significantly higher emissions of CO₂ were detected in the summer, while CH₄ emissions were maximum during spring. Cumulative CO₂ emissions by *C. papyrus* and *C. zizanioides* during the monitoring period showed similar trends with final values of about 775 and 1,074 g m⁻² respectively, whereas *M. giganteus* emitted 3,395 g m⁻². Cumulative CH₄ bed emission showed different trends for the three C₄ plant species in which total gas release during the study period was for *C. papyrus* 12.0 g m⁻² and ten times higher for *M. giganteus*, while *C. zizanioides* bed showed the greatest CH₄ cumulative emission with 240.3 g m⁻². The wastewater organic carbon abatement determined different C flux in the atmosphere. Gas fluxes were influenced both by plant species and monitored months with an average C-Emitted:C-Removed ratio for *C. zizanioides*, *C. papyrus* and *M. giganteus* of 0.3, 0.5, and 0.9 respectively. The growing season C balances were positive for all vegetated beds with the highest C sequestered in the bed with *M. giganteus* (4.26 kg m⁻²) followed by *C. zizanioides* (3.78 kg m⁻²) and *C. papyrus* (1.89 kg m⁻²). To our knowledge this is the first paper that present preliminary results on CO₂ and CH₄ emissions from CWs vegetated with C₄ plant species in Mediterranean basin during vegetative growth.

Introduction

Constructed wetlands (CWs) are natural-like systems widely used for wastewater treatment (Bulc 2006; Kadlec and Wallace, 2008; Bulc and Ojstršek, 2008; Barbera et al. 2009; Verlicchi and Zambello, 2014). CWs are characterized by high multifunctionality because in addition to wastewater depuration they provide reclaimed water for irrigation of crops and green areas (Cirelli et al. 2007, 2012) and produce biomass (Borin et al. 2013a) that can be harvested and used for energy purposes (Ciria et al. 2005). CW are
increasingly widespread for wastewater treatment in small communities and households where in addition to the fundamental purifying function, they also have decorative function that imposes the choice of plants characterized by high functional, amenity, and aesthetic values (Ghermandi et al. 2010). CWs carbon (C) cycles contribute to the global greenhouse gases (GHGs) balance through their carbon dioxide (CO$_2$) and methane (CH$_4$) emissions. In particular they can act as CO$_2$ sinks by photosynthetic CO$_2$ assimilation from the atmosphere or as a source of CO$_2$ through bed respiration (Barbera et al. 2014) and/or organic matter fermentation (CH$_4$) (Brix et al. 2001). Several variables, summarized into two categories, affect CO$_2$ and CH$_4$ emission: 1) abiotic variables such as CW age (Liikanen et al. 2006; Zemanova et al. 2010) and type (Liu et al. 2009; VanderZaag et al. 2010), water table level (Mander et al. 2005), wastewater quality, water flow and temperature (Johansson et al. 2004; Søvik and Kløve 2007; Zhu et al. 2007), climatic conditions (Mander et al. 2005; Søvik et al. 2006), sediment and wastewater redox status (Whalen and Reeburg 2000; Wang et al. 2008b; Zhou et al. 2008) and oxygen availability in the system (Maltais-Landry et al. 2009a); 2) biotic variables such as the presence and vascular plants species composition (Ström et al. 2007; Picek et al. 2007; Wang et al. 2013), plant biomass yield (Kao-Kniffin et al. 2010) and plant management (Zhu et al. 2007).

Plants presence influence the CO$_2$ (Ström et al. 2005) and CH$_4$ production and consumption (Segers 1998; Ström et al. 2005; Wang et al. 2008b) through their root systems (Lai et al. 2011), mainly with oxygen release (Jackson and Armstrong 1999) and roots exudate production (Saarnio et al. 2004). Furthermore in CWs vegetated with macrophyte, as Phragmites australis, the dominant mechanism of CH$_4$ release to the atmosphere is mediated by aerenchimatic plant tissue gas transport in the plants aerenchimatic tissue, primarily by pressurized convective gas flow (Brix et al. 2001). Therefore plant species with different anatomy and physiology and so different oxygen (Wigand et al. 1997) and exudate release levels (Ström et al. 2003) determine different CO$_2$:CH$_4$ ratio emission and global warming potential (GWP), considering that CH$_4$ has a 25 times higher effect on GWP than CO$_2$ (IPCC 2007). With this in mind, although C3 are the most utilize machrophyte as P. australis in CWs, C4 plant species although not resulting significant differences in CWs wastewater pollutant removal they could, with their
different physiology, affect the quantity of carbon dioxide equivalent (\(\text{CO}_2(\text{eq})\)) released to the atmosphere. Moreover the C4 plant have a relatively high potential of producing biomass from solar energy, which is one of the criteria for the selection of plants to be used in constructed wetlands (Perbangkhem and Polprasert, 2010).

The GHGs emissions and C balance have been studied and/or estimated for several natural wetlands worldwide (Bernal and Mitsch 2008, 2012; Mitsch et al. 2013) but only a few studies have been done on the C balance and plant species effect in CWs (Meuleman et al. 2003; Picek et al. 2007; Mander et al. 2008; de Klein and van der Werf 2014). Furthermore although \(\text{CO}_2\) is a major GHG, there are only a limited number of studies reporting on \(\text{CO}_2\) emissions in CWs (Mander et al. 2014a) and only one in Mediterranean areas (Garcia et al. 2007). The aim of this research was to study, during the first operating year, the \(\text{CO}_2\) and \(\text{CH}_4\) emissions and C budgets of a CW horizontal subsurface flow pilot-plant vegetated with three C4 plant species (\textit{Cyperus papyrus}, \textit{Chrysopogon zizanioides} and \textit{Mischantus giganteus}).

**Materials and Methods**

**Study site**

The research was conducted from April 1st to November 30th 2012 in a pilot plant located in San Michele di Ganzaria (Eastern Sicily – latitude 37°30′ North, longitude 14°25′ East, altitude 370 m a.s.l.). The area has a typical Mediterranean climate (Köppen climate classification) with rainfall of approximately 500 mm y\(^{-1}\), mainly in the winter. The pilot plant consists of three parallel horizontal subsurface flow (HSSF) beds, vegetated with either \textit{Cyperus papyrus} L. (papyrus), \textit{Chrysopogon zizanioides} (L.) Roberty (vetiver) or \textit{Mischantus x giganteus} Greef et Deu (mischantus). Each bed is rectangular in shape with a surface area of 4.5 m\(^2\) (1.5 m \(\times\) 3.0 m) and built in concrete, partially buried, and lined with an impermeable membrane. The beds were filled, to an average depth of 0.6 m, with volcanic gravel of 10–15 mm in size; during the monitoring period water depth was about 0.55 m. The size of each bed can be attributed to a size for a house with 4/5 Equivalent Inhabitants in an integrated household CW for domestic wastewater treatment. Wastewater inflow, monitored by flow meter, was 40 L h\(^{-1}\) during all the study period. The hydraulic retention time was about 22 hours. Further specifications
are given in (Toscano et al. 2009). *M. giganteus* and *C. zizanioides* were planted in December 2011, whereas *C. papyrus*, for logistical reasons, was planted in June 2012 with a density of 5.5 plants m$^{-2}$ with a relative influence on data magnitude.

**C4 Plants**

This work collates growth, productivity and performance information from various independent studies incorporating the three C4 plant species, belongs to Poaceae and Cyperaceae families, in constructed wetlands for wastewater treatment.

*Miscanthus x giganteus* Greef et Deu is a grass of the family Poaceae endemic to East Asian tropic and subtropic regions (Lewandowski et al. 2000), introduced in Europe as ornamental plant about 50 years ago (Lewandowski et al. 2003). It is well adapted to warmer climates (Venedaal et al. 1997) conditions of Central and South Italy (Angelini et al. 2009).

*Chrysopogon zizanioides* (L.) Roberty, commonly known as vetiver, is a fast-growing perennial tussock grass member of the family Poaceae, native to India, it is considered sterile outside its natural habitat. Vetiver grown worldwide for perfumery, agriculture, and bioengineering where it is used for soil and water conservation.

*Cyperus papyrus* L. is an herbaceous rhizomatous aquatic macrophyte inhabiting subtropical and tropical wetlands of the family Cyperaceae, commonly referred to as papyrus, it is native to Africa, and present at the lower latitude of Mediterranean Basin as in Sicily. Papyrus is mainly used in the African continent in the constructed wetland.

**Environmental parameters**

A CR510 automatic weather station (Campbell Scientific, Logan, UT) was installed close to the pilot plant to measure rainfall, air temperature, wind speed and global radiation. Evapotranspiration (ET) rates were estimated using a water balance method, i.e. measuring, for each bed, the influent wastewater flow rate, the water increase due to precipitation and the discharged wastewater volume.

**Gas sampling and analyses**

CO$_2$ and CH$_4$ sampling and analysis were done out from April 1$^{st}$ (start of vegetative cycle) to November 30$^{th}$ 2012 (end of vegetative cycle) for *M. giganteus* and *C. zizanioides*
whereas from June 1st (transplantation) to November 30th 2012 (end of vegetative cycle) for C. papyrus. Therefore in our study we calculate the plants growing season C budget. CO₂ emissions were estimated in situ using the static-stationary chamber technique. The cylindrical PVC chambers, two in each bed, were 35 cm in height and 12.5 cm wide. The bottom part (20 cm) was permanently inserted in the gravel substrate and the chamber was sealed with a lid in which the CO₂ emitted from the bed was retained in a sodium hydroxide (NaOH) solution trap (Barbera et al. 2014).

Considering that alkali trap used to measure CO₂ emission can underestimate the real gas flux (Jensen et al. 1996), in order to reduce the experimental error, to acceptable levels, we have made the following enhancements: 1) the trap solution was replaced every ten days to avoid any daily influence on the CO₂ flux estimate and to reduce the CO₂ atmospheric influence when the static chamber was open to replace the trap so the monthly total beds respiration (respiration of bed microbes, roots and rhizomes) was calculated based on a decadal dataset; 2) NaOH solution was maintained under 70% of its CO₂ absorption capacity, in agreement with Sharkov (1984), who reported no change in the absorption rate when the alkali was used until 70%; 3) to accelerate CO₂ absorption, a high jar/chamber surface ratio was carried in order to reduce the underestimated CO₂ measures at high soil respiration rates (above 300 mg m⁻² h⁻¹) (Yim et al. 2002).

CH₄ flux was measured using the static non-stationary chamber technique (Di Bella et al. 2011) three times a month in two points in order to replicate the measures. The flux cylindrical chamber, 42 cm high and 20 cm wide, was inserted into the gravel substrate during the measuring period using a permanent ring inserted into substrate three weeks before the beginning of measurements to prevent soil disturbance in each site. The surface CH₄ flow was determined by measuring the temporal change in CH₄ concentration inside the chamber using a portable FID (Crowcon Gas-Tec®) detecting CH₄ concentrations at levels of parts per million. CH₄ flux was calculated using the following formula:

\[
CH₄ = \frac{V}{A} \cdot \frac{dc}{dt}
\]
where CH\textsubscript{4} flux is expressed in mg CH\textsubscript{4} m\textsuperscript{-2} s\textsuperscript{-1}; V (m\textsuperscript{3}) is the volume and A (m\textsuperscript{2}) the footprint of the flux chamber; ‘c’ is the CH\textsubscript{4} concentration (mg CH\textsubscript{4} m\textsuperscript{3}) and ‘t’ represents the time step (s). Data are considered acceptable according to the English Environment Agency (2007) guidelines criteria.

**Water sampling and analyses**

The various water physicochemical variables, total suspended solids (TSS) at 105°C, COD, total nitrogen (TN) and ammonium (NH\textsubscript{4}\textsuperscript{+}-N) were evaluated monthly at the inlet and outlet of each bed according to APHA (1998) methods with two samples in the same day. The evaluation of treatment performance was based on the removal efficiency percentage calculated using average inflow and outflow wastewater concentrations and volumes.

**Biomass sampling and analyses**

In December 2012 aboveground and belowground biomass were sampled at three points in each of the beds. The belowground biomass was taken at three depths (0-20, 20-40 and 40-60 cm) and manually divided into roots and rhizomes. Biomass subsamples were homogenized for quality analysis and dry weight was determined by drying the biomass in a thermo-ventilated oven at 65 °C until constant weight was reached; biomass fiber composition was determined using the procedures of Goering and Van Soest (1970); biomass C content was determined by CNS Macrovario combustion analyzer (Elementar Analysensysteme GmbH, Germany).

**Carbon balance calculation during the growing season**

Carbon balance was calculated using the follow equation:

\[
\text{C balance} = \text{WC}_{\text{in}} + \text{ABC}_{\text{fix}} + \text{BBC}_{\text{fix}} - \text{GHGsC}_{\text{out}} - \text{WC}_{\text{out}}
\]

where WC\textsubscript{in} is the C inputs due to the inflowing wastewater, ABC\textsubscript{fix} is the C fixed in the plants aboveground biomass, BBC\textsubscript{fix} is the C fixed in the plants belowground biomass, GHGsC\textsubscript{out} is the C emitted as CO\textsubscript{2} and CH\textsubscript{4} during the growing season, and WC\textsubscript{out} is the C outputs due to the outflowing wastewater. Carbon concentration in inflow and outflow wastewater was calculated based on COD concentrations. COD was converted to C using the coefficients 0.31 and 0.36 for the inflowing and outflowing water respectively.
(Pitter 1999). C content in the plants biomass was calculated using C concentration determined as reported in section 2.5 (CNS data). C emissions from the bed surface were calculated as the sum of CO2-C and CH4-C measured as described in section 2.3. In agreement with Mander et al. (2008), the aboveground biomass plant respiration was not considered, because the C respired was first assimilated by plant gross photosynthesis. The GWP of the studied systems was calculated by converting the fluxes of CH4 into 25 CO2 equivalents (IPCC 2007).

**CO2(eq) balance during the growing season**

Carbon environmental balance was calculated considering the GHGs emission and plants biomass C fixed for each gram of C removed from wastewater and, CO2, CH4 and C biomass are expressed in terms of CO2(eq) using the following formula:

\[
\text{CO2(eq)} = C_{\text{biomass}} \times \frac{44}{12} - \text{CO2} - (\text{CH4} \times 25)
\]

where CO2 and CH4 were the GHGs emission measured during the growing season; CH4 emissions was computed as 25 times CO2 (IPCC 2007). CO2(eq) due to C biomass was computed multiply this last for 44/12, the value 44 represents the molecular weight of CO2, and the value 12 is the atomic weight of C.

**Statistical analysis**

In our experimental design we use replication through time and space rather than through independent experimental units. The normality of data was checked using the Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk tests. CO2 and CH4 emissions from the study sites didn’t show normal distribution, so the Kruskal–Wallis non-parametric test was used to check the significance of differences (accepted at the level of p<0.05). Correlation between average air temperature and solar radiation with CO2 and CH4 emissions were evaluated using Spearman Rank correlation.

**Results**

**Environmental parameters**

Meteorological data recorded at the site during the monitoring period (April–November 2012) are reported in figure 1. Air temperature reached the maximum value on July 13th
(43.4 °C) and minimum on April 1st (1.5 °C), the highest value of average solar radiation (27.6 MJ m\(^{-2}\) d\(^{-1}\)) was recorded in July.

**Figure 1** – Meteorological data recorded in San Michele di Ganzaria during study period.

ET measurements (Fig. 2) showed very high values, close to 14 mm d\(^{-1}\) in August for *C. zizanoides*, confirming the strong effect of vegetation in CW systems, as reported by Borin et al. (2011).
The correlation between average air temperature and solar radiation with GHGs emissions showed a species-specific response. *C. zizanioides* bed showed positive correlations of average air temperature and solar radiation with CO$_2$ and CH$_4$ emissions respectively, *M. giganteus* only for CH$_4$ emission with solar radiation. *C. papyrus* showed a positive result for all the correlations studied but this should be considered in relation to the shorter vegetative period (Tab.1).

**Table 1** – CO$_2$ and CH$_4$ beds emission correlation with average air temperature and solar radiation.

<table>
<thead>
<tr>
<th>Correlation</th>
<th><em>C. zizanioides</em></th>
<th><em>C. papyrus</em></th>
<th><em>M. giganteus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CO$_2$ vs Average air T$^\circ$</td>
<td>0.891***</td>
<td>0.664**</td>
<td>0.310 n.s.</td>
</tr>
<tr>
<td>CO$_2$ vs Solar radiation</td>
<td>0.255 n.s.</td>
<td>0.721**</td>
<td>-0.363 n.s.</td>
</tr>
<tr>
<td>CH$_4$ vs Average air T$^\circ$</td>
<td>0.295 n.s.</td>
<td>0.535*</td>
<td>0.042 n.s.</td>
</tr>
<tr>
<td>CH$_4$ vs Solar radiation</td>
<td>0.861***</td>
<td>0.622*</td>
<td>0.795***</td>
</tr>
</tbody>
</table>

n.s. = not significant; * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001

**Water analysis**

The beds wastewater treatment performance, based on removal efficiency percentage, are reported in table 2. In particular, the CWs resulted very efficient in the removal of TSS, showing average values higher than 81% and effluent concentrations always lower than 10 g m$^{-2}$ d$^{-1}$. The nitrogen showed a removal rate ranging from 46 to 59% (TN) and from 40 to 54% (NH$_4^+$), with higher values obtained in the system planted with *C. zizanioides*. 
Table 2 – Beds percentage removal efficiency.

<table>
<thead>
<tr>
<th>Variables</th>
<th>UM</th>
<th>C. zizanioides</th>
<th>C. papyrus</th>
<th>M. giganteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS</td>
<td>%</td>
<td>90.2 ± 9.6</td>
<td>81.2 ± 24.7</td>
<td>90.3 ± 7.0</td>
</tr>
<tr>
<td>TN</td>
<td>%</td>
<td>59.4 ± 13.4</td>
<td>45.7 ± 19.2</td>
<td>57.2 ± 16.3</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>%</td>
<td>51.8 ± 20.7</td>
<td>40.0 ± 28.7</td>
<td>54.3 ± 23.5</td>
</tr>
</tbody>
</table>

On average COD monthly removal ranged from about 55% to 65% between the three studied beds (figure 3).

![Figure 3 – COD influent vs effluent wastewater contents.](image)

Biomass production and characteristics

At the end of the vegetative season, total dry plant biomass production was highest in *M. giganteus* (7.4 kg m⁻²), followed by *C. zizanioides* (5.3 kg m⁻²) and *C. papyrus* (1.8 kg m⁻²). The relative papyrus reduced biomass production was partially due to the delay in plants transplanting. The highest aboveground and root biomass production was measured in *C. zizanioides*; *M. giganteus* showed the highest total belowground biomass, mainly constituted of rhizomes lying in the first 20 cm (Tab.3). Root density declined with depth, markedly in *C. zizanioides* with only 2.6% of root presence in the 20-40 cm layer (0.3 kg m⁻²); whereas *C. papyrus* and *M. giganteus* showed 27.6% and 78.1% of root production in the deeper layer respectively (Tab.3). At the end of the study period *C. zizanioides* showed the highest aboveground:belowground ratio among
the three species and the greatest concentration of roots in the bed aerobic layer (0-20 cm; Tab.3).

**Table 3 – Aboveground and belowground biomass production at the end of the study period and plants fraction incidence.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (kg m⁻²)</th>
<th>Belowground biomass (kg m⁻²)</th>
<th>Aboveground: Belowground ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aboveground biomass (kg m⁻²)</td>
<td>Roots</td>
<td>Roots</td>
</tr>
<tr>
<td>C. papyrus</td>
<td>1.10 ± 0.13</td>
<td>0.2 ± 0.02</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>C. zizanioides</td>
<td>4.95 ± 0.08</td>
<td>0.31 ± 0.03</td>
<td>0.01 ± 0.001</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>4.02 ± 1.05</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.003</td>
</tr>
</tbody>
</table>

*the rhizomes were located close to the bed surface

**CO₂ and CH₄ emissions**

Significantly different GHGs (CO₂ and CH₄) values and ratio emissions were measured in the studied plant species and over the monitoring period.

The CO₂ emission was significantly higher during the summer season (Fig.4a). The monthly CO₂ average daily emission ranged between 1.5 ± 0.9 and 27.0 ± 6.6 g m⁻² d⁻¹ respectively for C. zizanioides in April and M. giganteus in September. Considering the plant species, on the average of the seasons, a significantly lower emission of CO₂ was observed in C. papyrus and C. zizanioides, with a median average value of 3.8 g m⁻² d⁻¹, compared to the bed with M. giganteus that showed a median value 2.8 times higher (Fig.5a). In our study C. papyrus and C. zizanioides showed lower values of CO₂ cumulative emissions, with about 775 and 1,074 g m⁻² respectively, than M. giganteus (3,395 g CO₂ m⁻²).

Considering the CH₄ fluxes, they were significantly highest in the spring followed by summer and fall (Fig. 4b). The highest value of CH₄ emission was measured in June from the C. zizanioides bed. Instead negligible emissions were detected in October and November for all beds. Considering the CH₄ emission for the three species, no statistical differences were found between C. zizanioides and M. giganteus, whereas significantly lower emissions were detected for C. papyrus bed which, however, was planted in June (Fig.5b). In December only for the bed vegetated with M. giganteus after plant cutting (data not show), for fourteen days, CH₄ emissions was detected. Cumulative estimate CH₄ emission during the study period was 12.0 g m⁻² for C. papyrus, 121.1 g m⁻² for M. giganteus and 240.3 g m⁻² for C. zizanioides. The papyrus lower emission is justify by its lower growth due to the delay in plant transplanting.
Figure 4 – Box-plot diagrams of carbon dioxide (a) and methane (b) beds emission in different seasons. Different letters indicate significant differences at $p < 0.05$ by Kruskal–Wallis test.
Carbon balance during growing season

In terms of carbon balance (Tab.4), the higher C quantity was fixed in the bed vegetated with *M. giganteus* (4.3 kg m$^{-2}$), followed by *C. zizanioides* (3.8 kg m$^{-2}$) and *C. papyrus* (1.9 kg m$^{-2}$). Since CWs are multiyear operating wastewater depuration systems where the C fixed in the plants belowground biomass, after the settlement phase, remains stable as microbial biomass due to the root systems turnover, we have not include it (column$^5$)
in the C balance computing. Therefore considering the aboveground plant biomass management, with one yearly cut, which determined a C removal from the beds, the C balance for the three C4 plants shows that the higher C fixing was reached for *C. zizanioides* (3.7 kg m\(^{-2}\)), followed by *M. giganteus* (2.8 kg m\(^{-2}\)) and *C. papyrus* (1.6 kg m\(^{-2}\)).

**Table 4** – Beds carbon balance (kg m\(^{-2}\)).

<table>
<thead>
<tr>
<th>Species</th>
<th>GHGs(C_{\text{out}})(^{(1)})</th>
<th>WC(\text{in})(^{(2)})</th>
<th>WC(\text{out})(^{(3)})</th>
<th>ABC(\text{fix})(^{(4)})</th>
<th>BBC(\text{fix})(^{(5)})</th>
<th>C balance ((2+4+5) – (1+3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. zizanioides</td>
<td>0.47</td>
<td>3.09</td>
<td>1.06</td>
<td>2.10</td>
<td>0.12</td>
<td>3.78</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>1.02</td>
<td>3.09</td>
<td>1.07</td>
<td>1.81</td>
<td>1.45</td>
<td>4.26</td>
</tr>
<tr>
<td>C. papyrus</td>
<td>0.22</td>
<td>2.26</td>
<td>0.82</td>
<td>0.41</td>
<td>0.26</td>
<td>1.89</td>
</tr>
</tbody>
</table>

GHGs\(C_{\text{out}}\) = C emitted as CO\(_2\) and CH\(_4\); WC\(\text{in}\) = C inputs with inflowing wastewater; WC\(\text{out}\) = C outputs with the outflowing wastewater; ABC\(\text{fix}\) = C fixed in the plants aboveground biomass; BBC\(\text{fix}\) = C fixed in the plants belowground biomass.

**CO\(_2\)\(\text{eq}\) balance during growing season**

The highest monthly daily average GWP (69.9 g m\(^{-2}\) d\(^{-1}\) CO\(_2\)\(\text{eq}\)) was computed in June for *C. zizanioides* (Tab.5). At the end of the eight months of monitoring *C. zizanioides* and *M. giganteus* beds had similar cumulative GWP of 7,082.0 and 6,423.0 g CO\(_2\)\(\text{eq}\) m\(^{-2}\) respectively, although with different trends; *C. papyrus* instead had a cumulative GWP of 1,075.6 g CO\(_2\)\(\text{eq}\) m\(^{-2}\). From *C. papyrus* transplanting (July) to November, *M. giganteus* showed more than twice the CO\(_2\)\(\text{eq}\) cumulative emission than those calculated for *C. zizanioides* and *C. papyrus*, which presented the same trend (Fig.6). Although this results are indubitably interesting they should be considered taking into account that, in this research, the internal plants gas transport, by convective flow fluxes, was not considered. The growing season cumulative GWP trend suggests a species-specific and environmental effect for removal of organic matter contained in the inflow wastewater.

In fact considering CO\(_2\)\(\text{eq}\) emission for each gram of C removed from the wastewater, on the average of the monitoring period, an emission was measured of 0.7, 3.5 and 3.2 g CO\(_2\)\(\text{eq}\) m\(^{-2}\) for *C. papyrus*, *C. zizanioides* and *M. giganteus* respectively. Considering the biomass C content (Tab.6), *C. papyrus*, *C. zizanioides* and *M. giganteus* fixed from the atmosphere, for each gram of C removed from the wastewater, 1.7, 4.0 and 5.9 g CO\(_2\) m\(^{-2}\) respectively, showing a positive CO\(_2\)\(\text{eq}\) balance.
Table 5 – Monthly carbon reduced in the wastewater and emitted in the atmosphere.

<table>
<thead>
<tr>
<th>Species</th>
<th>Months</th>
<th>C Influent (g m(^{-2}) d(^{-1}))</th>
<th>C Effluent (g m(^{-2}) d(^{-1}))</th>
<th>C-removed (g m(^{-2}) d(^{-1}))</th>
<th>CO(_2)-C Emitted (g m(^{-2}) d(^{-1}))</th>
<th>CH(_4)-C Emitted (g m(^{-2}) d(^{-1}))</th>
<th>C-Emitted: COD-removed</th>
<th>CO(_2)(eq) emission (g m(^{-2}) d(^{-1}))</th>
<th>CO(_2)(eq): COD-removed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. papyrus</strong></td>
<td>July</td>
<td>13.2 ± 2.0</td>
<td>9.3 ± 0.9</td>
<td>4.0</td>
<td>1.78 ± 0.17</td>
<td>0.14 ± 0.12</td>
<td>1.9</td>
<td>0.11</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>11.9 ± 1.1</td>
<td>9.9 ± 2.2</td>
<td>2.0</td>
<td>1.69 ± 0.36</td>
<td>0.10 ± 0.03</td>
<td>1.8</td>
<td>0.17</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>7.6 ± 1.6</td>
<td>2.8 ± 0.8</td>
<td>4.7</td>
<td>1.61 ± 0.30</td>
<td>0.05 ± 0.09</td>
<td>1.7</td>
<td>0.10</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>19.2 ± 3.5</td>
<td>1.9 ± 0.6</td>
<td>17.3</td>
<td>0.80 ± 0.22</td>
<td>0.00 ± 0.00</td>
<td>0.8</td>
<td>0.01</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>21.9 ± 5.5</td>
<td>2.6 ± 0.8</td>
<td>19.4</td>
<td>1.03 ± 0.26</td>
<td>0.00 ± 0.00</td>
<td>1.0</td>
<td>0.02</td>
<td>3.9</td>
</tr>
<tr>
<td><strong>C. zizanoides</strong></td>
<td>April</td>
<td>11.8 ± 2.1</td>
<td>5.5 ± 0.8</td>
<td>6.3</td>
<td>0.41 ± 0.24</td>
<td>0.40 ± 0.28</td>
<td>0.8</td>
<td>0.04</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>8.5 ± 1.2</td>
<td>7.0 ± 1.3</td>
<td>1.5</td>
<td>0.80 ± 0.09</td>
<td>1.72 ± 0.44</td>
<td>2.5</td>
<td>0.32</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>7.0 ± 0.8</td>
<td>2.9 ± 0.6</td>
<td>4.1</td>
<td>1.20 ± 0.18</td>
<td>1.97 ± 0.65</td>
<td>3.2</td>
<td>0.22</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>13.2 ± 2.0</td>
<td>4.5 ± 1.2</td>
<td>8.8</td>
<td>2.15 ± 1.01</td>
<td>1.33 ± 0.15</td>
<td>3.5</td>
<td>0.11</td>
<td>52.2</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>11.9 ± 1.1</td>
<td>6.2 ± 0.9</td>
<td>5.7</td>
<td>1.64 ± 0.15</td>
<td>0.28 ± 0.13</td>
<td>1.9</td>
<td>0.09</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>7.6 ± 1.6</td>
<td>3.6 ± 0.9</td>
<td>4.0</td>
<td>1.56 ± 0.17</td>
<td>0.08 ± 0.09</td>
<td>1.6</td>
<td>0.11</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>19.2 ± 3.5</td>
<td>2.1 ± 0.7</td>
<td>17.2</td>
<td>1.01 ± 0.07</td>
<td>0.11 ± 0.19</td>
<td>1.1</td>
<td>0.02</td>
<td>7.3</td>
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<td></td>
<td>November</td>
<td>21.9 ± 5.5</td>
<td>2.8 ± 1.0</td>
<td>19.2</td>
<td>0.81 ± 0.26</td>
<td>0.01 ± 0.01</td>
<td>0.8</td>
<td>0.01</td>
<td>3.2</td>
</tr>
<tr>
<td><strong>M. giganteus</strong></td>
<td>April</td>
<td>11.8 ± 2.1</td>
<td>5.5 ± 1.4</td>
<td>6.5</td>
<td>0.49 ± 0.29</td>
<td>0.67 ± 0.31</td>
<td>1.2</td>
<td>0.05</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>8.5 ± 1.2</td>
<td>7.0 ± 0.8</td>
<td>1.9</td>
<td>2.40 ± 1.01</td>
<td>0.99 ± 0.48</td>
<td>3.4</td>
<td>0.38</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>7.0 ± 0.8</td>
<td>2.9 ± 0.8</td>
<td>4.3</td>
<td>3.88 ± 1.30</td>
<td>0.92 ± 0.56</td>
<td>4.8</td>
<td>0.32</td>
<td>44.9</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>13.2 ± 2.0</td>
<td>4.5 ± 1.4</td>
<td>9.4</td>
<td>2.32 ± 0.56</td>
<td>0.16 ± 0.07</td>
<td>2.5</td>
<td>0.08</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>11.9 ± 1.1</td>
<td>6.2 ± 1.1</td>
<td>4.8</td>
<td>4.57 ± 0.78</td>
<td>0.16 ± 0.02</td>
<td>4.7</td>
<td>0.25</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>7.6 ± 1.6</td>
<td>3.6 ± 0.8</td>
<td>3.6</td>
<td>7.36 ± 1.80</td>
<td>0.05 ± 0.05</td>
<td>7.4</td>
<td>0.56</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>19.2 ± 3.5</td>
<td>2.1 ± 0.8</td>
<td>17.0</td>
<td>6.26 ± 0.79</td>
<td>0.03 ± 0.02</td>
<td>6.3</td>
<td>0.11</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>21.9 ± 5.5</td>
<td>2.8 ± 1.0</td>
<td>18.8</td>
<td>3.06 ± 1.18</td>
<td>0.01 ± 0.01</td>
<td>3.1</td>
<td>0.05</td>
<td>11.4</td>
</tr>
</tbody>
</table>
Table 6 – Percentage carbon content in biomass fractions.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (%C)</th>
<th>Belowground biomass (%C)</th>
<th>Roots 0-20 cm</th>
<th>Roots 20-40 cm</th>
<th>Rhizomes 0-20 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. papyrus</td>
<td>37.05</td>
<td></td>
<td>38.54</td>
<td>40.29</td>
<td>41.39</td>
</tr>
<tr>
<td>C. zizanoides</td>
<td>42.49</td>
<td></td>
<td>38.87</td>
<td>38.49</td>
<td>------</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>45.08</td>
<td></td>
<td>39.91</td>
<td>41.58</td>
<td>43.29</td>
</tr>
</tbody>
</table>

Figure 6 – Cumulative CO$_2$(eq) emission trends for the three species.

**Discussion**

CO$_2$ emissions showed a plant species-specific link, in agreement with Verville et al. (1998), whom found that vegetation composition had a greater effect on CO$_2$ emissions than abiotic conditions. Maltais-Landry et al. (2009a) in HSSF CWs mesocosm vegetated with *P. australis*, *Typha angustifolia* or *Phalaris arundinacea* reported that both plant presence and species had a significant impact on CO$_2$ fluxes, detecting higher emission in the units vegetated with *T. angustifolia*. The previous results are partially in contrast with Ström et al. (2007) which found that plant species seemed to have little or no effect on the total ecosystem respiration, although they reported a significantly higher emission from vegetated than un-vegetated sites. Garcia et al. (2007) in anaerobic conditions in a HSSF CWs plan located in the Mediterranean areas vegetated with *P. australis* reported a CO$_2$ emission values varying from 0.11 to 0.49 g CO$_2$ m$^{-2}$ d$^{-1}$.
Considering the possible different seasonal emission patterns of CO₂ emissions, we found a significant higher emission in the summer season in agreement with Søvik et al. (2006) which, in a North Europe (Estonia, Norway and Poland) comparative study on GHG emissions from HSSF CWs, have reported an average CO₂ emissions varying between 2.53 ± 0.15 g m⁻² d⁻¹ (winter season) and 10.63 ± 0.59 g m⁻² d⁻¹ (summer season). Also Maltais-Landry et al. (2009a) in HSSF CWs reported higher fluxes during the summer season compared to fall and winter ones.

Concerning CH₄ highest spring fluxes were probably due to the plants settlement phase when root system development was fast with a release of high amounts of exudates that supply substrate for methanogen microorganisms (Ström et al. 2003; Saarnio et al. 2004). It could be supported by the positive correlation for all species between CH₄ emissions and solar radiation (Tab.1), which influences plant photosynthesis and so root exudates. Johansson et al. (2004) in a pilot scale CW in spring and fall seasons detected gas fluxes ranging from -375 mg m⁻² d⁻¹ to 1739 mg m⁻² d⁻¹, with summer flux ca. 10-50 fold higher than other growing seasons. Furthermore, the authors reported that the seasonal shift in CH₄ rates also seems to be related to changes in the substrate and water temperature, which contribute to explaining the variation in gas emission rates. de Klein and van der Werf (2014) measuring CH₄ emission in May in a CW covered for 90% of P. australis (110 stems m⁻²) at two water temperatures (15° and 24°C) reported an emission respectively of 187.2 and 588.0 mg CH₄ m⁻² d⁻¹. Stadmark and Leonardson (2005) studied the parameters that regulate the GHGs emission in ponds reporting that water temperature was a good predictor of CH₄ emission with emissions between 1 and 54 mg m⁻² h⁻¹ when water temperature were higher than 15°C, and less than 0.6 mg CH₄ m⁻² h⁻¹ when water temperature was below 10 °C. In fact under low temperature the substrates for methanogenesis are reduced and consequently CH₄ emission is dropped (Zhu et al. 2007). Søvik et al. (2006) and Wang et al. (2008b) found a significant higher CH₄ emission in the summer season compared with the other ones, confirming that in CWs CH₄ emission is greatly influenced by temperature which also acts on thermophilic processes, as plant photosynthesis and microbial activities, which affect the CH₄ emissions influencing the CH₄-oxidizing and CH₄-producing microbial communities and their level of activity (Moore and Dalva, 1993). CH₄ emissions detected for M.
giganteus immediately after plant harvest is in agreement with Zhu et al. (2007) who reported an effect of plant cutting on CH₄ emissions. This gas emission could be due to belowground rhizomes fiber composition (Tab.7) that determined a release of easily degradable organic carbon (hemicellulose and cellulose; Amougou et al. 2011). *C. papyrus* has rhizomes lying close to the bed surface, so they are unavailable for anoxic microbial degradation. In fact no CH₄ production was measured, although they had a high content of easily degradable biomass (Tab.7).

Table 7 – Plant fiber composition.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass % fiber composition</th>
<th>Roots biomass % fiber composition</th>
<th>Rhizomes biomass % fiber composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemicellulose</td>
<td>Cellulose</td>
<td>Lignin (ADL)</td>
</tr>
<tr>
<td><em>C. papyrus</em></td>
<td>31.9</td>
<td>28.9</td>
<td>4.8</td>
</tr>
<tr>
<td><em>M. giganteus</em></td>
<td>32.2</td>
<td>42.4</td>
<td>9.3</td>
</tr>
<tr>
<td><em>C. zizanoides</em></td>
<td>35.4</td>
<td>39.4</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Moreover the CWs CH₄ flux rate is determined by different aspects, but the influence of plant species is of major importance on biochemistry of C in CH₄ flux to/from the atmosphere (Wang et al. 2013). The differences in plants root and stem architecture, aerenchymous tissue, and oxygen availability for rhizospheric bacteria result in ultimate differences in the methanogens and methanotrophs biomass (Inamori et al. 2007) that, in our case, could determine the no significant different emissions between *M. giganteus* and *C. zizanoides*. In fact in our study both species showed the higher belowground biomass production in the first 20 cm of HSSF CWs. Inamori et al. (2007), in VSSF CW units vegetated with *Zizania latifolia* found that the 90% of the root biomass was concentrated in the upper 10 cm whereas in the *P. australis* bed the root biomass was more deeper and evenly distributed along the substrate profile. Furthermore the authors reported that in *Z. latifolia* units, the number of methanotrophs was mainly concentrated in the first 10 cm and then decreasing along the depth of the rhizosphere; on the contrary
P. australis units showed a smaller number of this bacteria in the first 10 cm layer and larger number in the deeper layer (20 and 30 cm).

Considering CO$_2$-C and CH$_4$-C emissions, the reduction of 1 g of COD from the wastewater determined different C fluxes in the atmosphere that was influenced by plant species and months (Tab.5). On the average of the study period C. zizanioides, C. papyrus and M. giganteus showed C-Emitted:C-Removed ratios of 0.3, 0.5 and 0.9 respectively. The higher C flux from the M. giganteus bed, given that the study was conducted during the first bed operating year was not due to organic matter accumulation but can be due to the higher biomass production. In agreement with Liikanen et al. (2006) that reported in ten years period CW study an increased C gas production due to the increased plant biomass. The ability to secrete various exudates compounds into the rhizosphere is one of the most remarkable metabolic features of plant roots which can range about from 5% to 21% of photosynthetically fixed C (Marschner 1995). Through exudates, plants can regulate the microbial communities structure in the surrounding rhizosphere (Walker et al. 2003; Nardi et al. 2000; Weber et al. 2008; Weber and Legge 2011). As reported by Picek et al. (2007), root exudates are easily decomposable, so they can be preferentially used by microorganisms and transformed into CO$_2$ and CH$_4$, increasing total GHGs emissions from the CW. M. giganteus produced 4.2 and 1.7 times more biomass than C. papyrus and C. zizanioides, with a 4.6 and 2.1 times higher C emission as GHGs respectively. Therefore in our experiment the higher C flux detected in M. giganteus bed than the other ones could be due to higher root exudates produced as consequence of higher biomass of this beds than the other ones.

**Conclusions**

This is the first paper that present preliminary results on CO$_2$ and CH$_4$ emissions from CWs sized for single households vegetated with C4 plant species in Mediterranean Basin during vegetative growth. Total plant biomass harvested was, as expected, different in the three species, with the highest amount produced by M. giganteus. C. zizanioides had the greatest aboveground biomass with the highest aboveground:belowground ratio, whereas M. giganteus had the greatest total belowground biomass. All species showed higher root system growth in the first 20 cm beds layer.
CO₂ and CH₄ emissions from the three vegetated beds showed different values during the monitoring period. *C. papyrus* and *C. zizanioides* gave lower values of CO₂ cumulative emissions, with about 775 and 1,074 g m⁻² respectively, than *M. giganteus* (3,395 g CO₂ m⁻²). The CH₄ fluxes were significantly higher during the spring months than during summer and fall. The highest emission was recorded in June (2.6 ± 0.9 g m⁻² d⁻¹) from the *C. zizanioides* bed. Plant cutting determined a methane emission only in the bed vegetated with *M. giganteus*. CH₄ cumulative emission showed different trends for *C. papyrus* and *M. giganteus* in which total gas release during the study period was 12.0 and 121.1 g m⁻² respectively, while the *C. zizanioides* bed showed the greatest cumulative emission of 240.3 g m⁻².

Vegetation management influenced the C balance. Although the higher C quantity was fixed in the bed vegetated with *M. giganteus* (4.3 kg m⁻²), followed by *C. zizanioides* (3.8 kg m⁻²) and *C. papyrus* (1.9 kg m⁻²), if plants belowground biomass is not considered, due to the stable root C turnover during the expected CWs operating years lifetime, the higher C fixing was reached for *C. zizanioides* (3.7 kg m⁻²), followed by *M. giganteus* (2.8 kg m⁻²) and *C. papyrus* (1.6 kg m⁻²). The cumulative GWP trend suggests a species-specific environmental effect for wastewater depuration, with final values of 7,082.0, 6,423.0 and 1,075.6 g CO₂(eq) m⁻² for *C. zizanioides*, *M. giganteus* and *C. papyrus* respectively. This study without considering the winter plant dormancy phase shown that all the C₄ vegetated HSSF beds, during plants growing season, act as CO₂ sink.
Chapter IV
Role of C3 plant species on CO$_2$ and CH$_4$ emissions in Mediterranean constructed wetland
Abstract

C3 plant species are widely used to vegetate constructed wetlands (CW), but up today no information are available on their effect on CWs CO$_2$(eq) balance in Mediterranean climate. The aim of this research was to study the carbon dioxide (CO$_2$) and methane (CH$_4$) emissions and CO$_2$(eq) budgets of CW horizontal sub-surface flow pilot-plant beds vegetated with *Arundo donax* L. and *Phragmites australis* (Cav.) Trin. ex Steud. compared with an unvegetated bed in Sicily. The highest total plant biomass production was measured in the bed vegetated with *A. donax* (17.0 kg m$^{-2}$), whereas *P. australis* produced 7.6 kg m$^{-2}$. CO$_2$ and CH$_4$ emissions showed significant correlation with average air temperature and solar radiation for each bed. The CO$_2$ emission values ranged from 0.8 ± 0.1 g m$^{-2}$ d$^{-1}$, for the unvegetated bed in April, to 24.9 ± 0.6 g m$^{-2}$ d$^{-1}$ for the bed with *P. australis* in August. The average CO$_2$ emissions of the whole monitored period were 15.5 ± 7.2, 15.1 ± 7.1 and 3.6 ± 2.4 g m$^{-2}$ d$^{-1}$ for *A. donax*, *P. australis* and unvegetated beds respectively. The CH$_4$ fluxes differed significantly over the monitored seasons, with the highest median value being measured during spring (0.963 g m$^{-2}$ d$^{-1}$). No statistical differences were found for CH$_4$ flux among studied beds. Cumulative estimate CH$_4$ emissions during the study period (from April to December) were 159.5, 134.1 and 114.7 g m$^{-2}$ for *A. donax*, *P. australis* and unvegetated beds respectively. CO$_2$(eq) balance showed that the two vegetated beds act as CO$_2$(eq) sinks while the unvegetated bed, as expected, acts as a CO$_2$(eq) source. Considering only the aboveground plant biomass in the CO$_2$(eq) budgets, *P. australis* and *A. donax* determined uptakes of 1.30 and 8.35 kg CO$_2$(eq) m$^{-2}$ respectively.

Introduction

C3 plant species are widely used to vegetate constructed wetlands (CWs) which are natural-like systems widely used (Vymazal, 2010) to treat different wastewaters: landfill leachate (Bulc, 2006), agriculture drainage and animal (Borin and Tocchetto, 2007; Borin et al., 2013b), textile (Bulc and Ojstršek, 2008), urban (Barbera et al., 2009), pesticides (O’Geen et al., 2010). In these systems the organic carbon fraction content in the wastewaters is mainly removed through volatilization, determining a positive flux of greenhouse gases (GHGs) such as CO$_2$ and CH$_4$ into the atmosphere. However, the
atmospheric CO\textsubscript{2} uptake by plants photosynthesis can balance CO\textsubscript{2(eq)} fluxes (Barbera et al., 2014a). In general plants, with their root systems (Lai et al., 2011), influence the CO\textsubscript{2} production (Ström et al., 2005) and CH\textsubscript{4} production and consumption (Segers, 1998; Ström et al., 2005; Wang et al., 2008b), mainly through roots oxygen (Griess et al., 1990) and exudate release (Brix et al., 2001). In particular, plant species with different physiology and so different magnitude of oxygen (Wigand et al., 1997) and exudate release (Ström et al., 2003) can determine a different CO\textsubscript{2}:CH\textsubscript{4} ratio emission and global warming potential (GWP) given that CH\textsubscript{4} has a GWP 25 times higher than CO\textsubscript{2} (IPCC, 2007). In the Mediterranean Basin, where temperatures in the late spring and summer are high, the use of C3 species in CWs could determine a different environmental C balance compared with the CWs sited at higher latitude. Several studies on GHGs emission, at different latitudes, include CW beds vegetated with P. australis, which is the most widely studied C3 species. Only a few studies have been carried out in a Mediterranean environment on GHGs emission from pilot-plant CWs (Garcia et al., 2007; Barbera et al., 2014a) or a full-scale CW (Barbera et al., 2014b) vegetated with P. australis or C4 plant species. To our knowledge no studies have focused on GHGs emission from CW vegetated with A. donax (C3 species) either in a Mediterranean environment or at other latitudes.

The question if wetlands act as sink or source of GHGs has recently grown of importance to assess more completely the ecosystem services that they provide (Mitsch et al., 2013) and address their management. Since vegetation is the key component of CWs, its role has to be better known.

In this contest the aim of this research was to compare the carbon dioxide (CO\textsubscript{2}) and methane (CH\textsubscript{4}) emissions and CO\textsubscript{2(eq)} budgets of CW horizontal sub-surface flow (HSSF) pilot-plant beds unvegetated or vegetated with Arundo donax L. and Phragmites australis (Cav.) Trin. ex Steud..

**Materials and Methods**

**Study site**

The research was conducted from April 1\textsuperscript{st} to December 20\textsuperscript{th} 2012 in a pilot plant located in San Michele di Ganzaria (Eastern Sicily – latitude 37°30’ North, longitude 14°25’
East, altitude 370 m a.s.l.). The area has a typical Mediterranean climate (Köppen climate classification), with medium rainfall (approximately 500 mm y$^{-1}$) mainly in the winter. The pilot plant consisted of three parallel horizontal subsurface flow (HSSF) beds, two vegetated with *Phragmites australis* (Cav.) Trin. ex Steud. (common reed) and *Arundo donax* L. (giant reed) and a control unvegetated. Each bed is rectangular in shape with a surface area of 4.5 m$^2$ (1.5 m × 3.0 m) and was built of concrete, partially buried, and lined with an impermeable membrane. The beds were filled, to an average depth of 0.6 m, with 10–15 mm volcanic gravel; during the monitoring period the water depth was about 0.55 m. System details are available in Toscano et al. (2009). Wastewater inflow was 40 L h$^{-1}$. Beds were planted in December 2011 at a density of 5.5 plants m$^{-2}$.

**Studied C3 species**

*P. australis* is the macrophyte most frequently used to vegetate CWs. It is a tall perennial grass of the Poaceae family found in natural wetlands throughout temperate and tropical regions of the world. It commonly forms extensive stands (known as reed beds) and is capable of reproduction by seed, but primarily asexually multiplication via rhizomes. *P. australis* is a C3 plant species characterized by aerating tissues (aerenchyma channels and pith cavities) which play a crucial role in this species and generally in aquatic plants by providing oxygen to the submerged organs and often to the rhizosphere (Engloner, 2009).

*A. donax* is a tall perennial herbaceous plant of the same family occurring in grasslands and wetlands over a wide range of climatic habitats. It is classified as an emergent aquatic plant (Cook, 1990). Like *P. australis*, it is a C3 species, with an unusually high photosynthetic capacity (Rossa et al., 1998) and productivity similar to those of C4 species (Christou et al., 2001; Borin et al., 2013a).

**Meteorological variables**

A CR510 automatic weather station (Campbell Scientific, Logan, UT) was installed close to the experimental plant to measure rainfall, air temperature, wind speed and global radiation.
Gas sampling and analyses

CO₂ and CH₄ sampling and analysis were performed from April 1ˢᵗ (vegetative regrowth) to December 20ˢᵗ 2012 (end of vegetative cycle). The gas samplers did not cover growing plants.

CO₂ emissions were estimated in situ using the static-stationary chamber technique. The cylindrical chambers, of PVC, were 35 cm in height and 12.5 cm wide. The bottom part (20 cm) was permanently inserted in the gravel substrate and the chamber was sealed with a lid in which the CO₂ emitted from the bed was absorbed in a sodium hydroxide (NaOH) solution trap following modifications reported in Barbera et al. (2014b) to reduce experimental error (Jensen et al., 1996). The CO₂ traps, two in each bed, were placed in the inner part of the beds to reduce the border effect. They were replaced every ten days so the monthly total beds respiration (respiration of bed microbes, roots and rhizomes) were calculated based on a decadal dataset.

CH₄ flux was measured using the static non-stationary chamber technique (Barbera et al., 2014a) three times a month in two microsites in order to replicate the measures. The flux cylindrical chamber, 42 cm high and 20 cm wide, was inserted into the gravel substrate using a permanent ring inserted into substrate before the beginning of measurements to prevent soil disturbance in each site. The surface CH₄ flow was determined by measuring the temporal change in CH₄ concentration inside the chamber using a portable FID (Crowcon Gas-Tec®) detecting CH₄ concentrations down to parts per million levels.

Biomass sampling and analyses

In December 2012 aboveground and belowground biomass were sampled from three points in the inner part of each bed in order to minimize the border effect. The belowground biomass was collected at three depths (0-20, 20-40 and 40-60 cm) and was divided in roots and rhizomes. Biomass subsamples were homogenized for quality analysis: biomass dry weight was determined using a thermo-ventilated oven at 65 °C until constant weight was reached. Biomass C content was determined by CNS Macrovario combustion analyzer (Elementar Analysensysteme GmbH, Germany).
Growing season CO$_2$(eq) balance

Carbon environmental balance was calculated considering the CO$_2$ and CH$_4$ emissions and the storage of fixed carbon in plant biomass in terms of CO$_2$(eq) using the following formula:

$$CO_2(\text{eq}) = C_{\text{biomass}} \times \frac{44}{12} - CO_2 - (CH_4 \times 25)$$

where CO$_2$ and CH$_4$ were the GHGs emission measured during the growing season; CH$_4$ emission for CO$_2$(eq) budgets was computed as 25 times CO$_2$ (IPCC, 2007); the value 44 represents the molecular weight of CO$_2$, and 12 is the C atomic weight. According to Mander et al. (2008) the aboveground plant biomass respiration was not considered, assuming that respired C was previously assimilated by plant gross photosynthesis.

Statistical analysis

The normality of data was tested using the Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk tests. CO$_2$ and CH$_4$ emissions from the study sites didn’t show normal distribution, so the Kruskal–Wallis non-parametric test was used to check the significance of differences (accepted at the level of p<0.05) using combination of replication in time and space rather than through independent experimental units. Correlations between average air temperature and solar radiation with CO$_2$ and CH$_4$ emissions were evaluated using Spearman Rank correlation. The distribution of range in biomass and emission values were expressed in terms of standard deviation.

Results and Discussion

Environmental parameters

Meteorological data recorded at the site during the monitoring period (April-December 2012) are reported in figure 1. Cumulative rainfall was 105.8 mm; the air temperature reached maximum value on July 13$^{th}$ (43.4 °C) and minimum on December 13$^{th}$ (-0.2 °C). The highest monthly average solar radiation value (27.6 MJ m$^{-2}$ d$^{-1}$) was recorded in July and average wind speed was generally below 1 m s$^{-1}$.
The correlation between average air temperature and solar radiation with CO$_2$ and CH$_4$ emissions showed a specific response for each bed (Tab.1). CO$_2$ emission was positively correlated with average air temperature in the bed vegetated with *P. australis* (p<0.001) instead no correlation was found for *A. donax*. For all beds CH$_4$ emission was positively correlated with solar radiation (p<0.001) whereas only the unvegetated bed showed positively correlation between CH$_4$ emission and average air temperature (p<0.05). The results are undoubtedly interesting, but caution must be used due to the short monitoring period.
period of about nine months and considering that the study was carried out during the first operating year and so the systems did not reach the belowground biomass turnover with a low amount of sludge accumulated in the beds. Both conditions could determine, in the following years, different emissions ratio between the two species and different response to environmental condition.

Table 1 – CO₂ and CH₄ beds emission correlation with temperature and solar radiation.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>P. australis</th>
<th>A. donax</th>
<th>Unvegetated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂ vs Solar radiation</td>
<td>0.108 n.s.</td>
<td>-0.311 n.s.</td>
<td>-0.541 **</td>
</tr>
<tr>
<td>CO₂ vs Average air Temperature</td>
<td>0.789 ***</td>
<td>0.293 n.s.</td>
<td>0.196 n.s.</td>
</tr>
<tr>
<td>CH₄ vs Solar radiation</td>
<td>0.760 ***</td>
<td>0.818 ***</td>
<td>0.798 ***</td>
</tr>
<tr>
<td>CH₄ vs Average air Temperature</td>
<td>0.191 n.s.</td>
<td>0.341 n.s.</td>
<td>0.477 *</td>
</tr>
</tbody>
</table>

n.s. = not significant; * = p < 0.05; ** = p < 0.01; *** = p < 0.001

Plants biomass production

At the end of the vegetative season, A. donax showed the higher total plant biomass yield (16.8 kg m⁻²) while P. australis produced 7.5 kg m⁻². Considering the plant organs biomass giant reed had always the highest production, namely 1.8, 5.1 and 3.3 times higher than common reed for aboveground, roots and rhizomes biomass respectively. Roots density declined with depth in the bed vegetated with A. donax, whereas for P. australis higher density was found in the 20-40 cm gravel layer. Rhizomes were distributed only in the first 20 cm of substrate for A. donax and in the 0-40 cm layer for P. australis (Tab.2). At the end of the study period P. australis showed higher aboveground/belowground ratio (2.7) than A. donax (1.5; Tab.2). The reported high aboveground production confirm the high productive potential of the two species cultivate under optimal water and nutritional availability condition (Idris et al., 2012a; Borin et al., 2013a).
Table 2 – Aboveground and belowground biomass production (±SD) at the end of the study period and plants fraction incidence.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (Mg ha(^{-1}))</th>
<th>Belowground biomass (Mg ha(^{-1}))</th>
<th>Roots: Rhizomes ratio</th>
<th>Aboveground:Belowground ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Roots 0-20 cm</td>
<td>Roots 20-40 cm</td>
<td>Roots 40-60 cm</td>
</tr>
<tr>
<td><em>P. australis</em></td>
<td>55.0 ± 3.9</td>
<td>0.24 ± 0.01</td>
<td>0.78 ± 0.06</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td><em>A. donax</em></td>
<td>99.3 ± 6.9</td>
<td>3.25 ± 0.16</td>
<td>2.01 ± 0.13</td>
<td>0.57 ± 0.08</td>
</tr>
</tbody>
</table>
GHGs emission

The average CO$_2$ emission, was significantly lower during the spring for all studied CWs beds, (Fig.2a) with the lowest monthly CO$_2$ average daily emission recorded in April with $5.2 \pm 1.6$, $6.1 \pm 1.0$ and $0.8 \pm 0.1$ g m$^{-2}$ d$^{-1}$ for common reed, giant reed and unvegetated beds respectively. The highest monthly average daily CO$_2$ emissions were recorded in August for common reed ($24.9 \pm 0.6$ g m$^{-2}$ d$^{-1}$) and in September for giant reed ($24.3 \pm 2.7$ g m$^{-2}$ d$^{-1}$) and the unvegetated bed ($6.6 \pm 1.1$ g m$^{-2}$ d$^{-1}$). On the average of the seasons, no significant differences were found in CO$_2$ emission between A. donax and $P.$ australis beds, instead a significantly lower emission was recorded from the unvegetated one (Fig.2b), with a median value 4.3 times lower than the average median value of vegetated beds. Bed respiration did not show species-specific effect, but there was a significantly higher emission from vegetated beds than the unvegetated one, confirming that the presence of vegetation is of great importance for CWs total ecosystem respiration (Ström et al., 2007). Nevertheless the effect of different species may be available as suggested by Maltais-Landry et al. (2009a) who reported significant differences among HSSF CW mesocosms vegetated with $P.$ australis, Phalaris arundinacea and Typha angustifolia.

In our study the average beds respiration, considering the whole monitored period, were $15.1 \pm 7.1$, $15.5 \pm 7.2$ and $3.6 \pm 2.4$ g CO$_2$ m$^{-2}$ d$^{-1}$ for $P.$ australis, A. donax and unvegetated beds respectively, in agreement with Barbera et al. (2014b) who reported, in the same area, from a full scale HSSF CW higher CO$_2$ emissions from vegetated sites than unvegetated one. Even at higher latitudes of the Southern Sweden, Ström et al. (2007) reported from a peat-based CW site, an average CO$_2$ flux of $25.1 \pm 4.7$ and $4.3 \pm 0.7$ g m$^{-2}$ d$^{-1}$, from a zone vegetated with $P.$ australis and unvegetated zone respectively. Søvik et al. (2006), in a Northern Europe (Estonia, Norway and Poland) comparative study on GHGs emission from vegetated HSSF CWs ($P.$ australis, Iris pseudocorus, Typha latifolia, and Scirpus lacustris), reported an average CO$_2$ emission of $2.5 \pm 0.2$ and $10.6 \pm 0.6$ g m$^{-2}$ d$^{-1}$ for winter and summer respectively. Picek et al. (2007), in an HSSF CW bed vegetated with $P.$ australis that treated combined sewage and stormwater runoff, reported CO$_2$ emissions varying between 0.4 and 27.2 g m$^{-2}$ d$^{-1}$ during summer and fall. In our study common and giant reed showed similar CO$_2$ cumulative emissions,
with about 3.98 and 4.08 kg m\(^{-2}\) respectively, whereas it was 0.94 kg m\(^{-2}\) for the unvegetated bed.

![Box-plot diagrams of carbon dioxide beds emissions in different seasons (a) and with different species (b). Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

The fluxes of CH\(_4\) were significantly different among the studied seasons (Fig.3a). The highest median value was measured during the spring (0.963 g m\(^{-2}\) d\(^{-1}\)), followed by summer (0.399 g m\(^{-2}\) d\(^{-1}\)) and fall (0.018 g m\(^{-2}\) d\(^{-1}\)). The highest CH\(_4\) spring emission was
probably due to the plants settlement phase characterized by: 1) a fast root system development resulting in high exudates release that improved methanogen microorganisms activities (Brix et al., 2001; Ström et al., 2003; Saarnio et al., 2004) in vegetated beds. This is supported by the positive correlation between CH₄ emissions and solar radiation, which influenced root exudates by photosynthesis activity (Grayston et al., 1997); 2) the incomplete root system development that determined less oxygen presence in the bed and so lower CH₄ oxidation rate. Considering the unvegetated bed, the significant (p<0.001) positive correlation between CH₄ emission and solar radiation can be supported by the indirect effect of this last on substrate and water temperature. Furthermore only for the unvegetated bed CH₄ emissions were also correlated (p<0.05) with average air temperature, in agreement with Tanner et al. (1997) who found a correlation between air temperature and CH₄ emissions, and with Sorrel et al. (1997) who reported significantly lower methanogenesis at 12 °C than 30 °C. Johansson et al. (2004) studying CH₄ emission from ponds which treated urban wastewater, reported that sediment and water temperatures explained a large proportion of the flux variations (33–43%). No statistical differences were found for the CH₄ emissions from the three studied beds (Fig.3b). Inamori et al. (2007) reported that CH₄ emission from plant units is the net balance between methanogens CH₄ production and methanotrophs oxidation to CO₂. Given that macrophytes’ aerenchymatous tissues transport and release oxygen into the rhizosphere, they increase CH₄ oxidation in the anaerobic bed layers (Griess et al., 1990; Jespersen et al., 1998; McDonald et al., 2002).

Cumulative estimating CH₄ emission during the study period was 159.5, 134.1 and 114.7 g m⁻² for A. donax, P. australis and the unvegetated bed respectively. The higher bed respiration and methane emissions from vegetated beds can be also attributed at more labile carbon being accessible via plant exudates (Zemanovà et al., 2010), estimated as 20% of aboveground biomass produced during growing season (Picek et al., 2007), that intensified bacterial activity (Gagnon et al., 2007). Ström et al. (2003) reported that CH₄ emission rates, and the potential CH₄ production, are dependent on substrate quality and the linkage between root exudation of labile carbon, e.g. acetate and CH₄ formation. estimate the root exudate as.
Figure 3 – Box-plot diagrams of methane beds emissions in different seasons (a) and with different species (b). Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

**Growing season CO$_2$(eq) balance**

The highest monthly average daily CO$_2$(eq) emission was computed for all beds in June ranged from 25.9 g m$^{-2}$ d$^{-1}$ (unvegetated bed) and 60.7 g m$^{-2}$ d$^{-1}$ (bed vegetated with *A. donax*). At the end of the trial period the two vegetated beds had similar CO$_2$(eq) cumulative emission values and trends (Fig.4), with 7.34 and 8.07 kg CO$_2$(eq) m$^{-2}$ for common and giant reed respectively. The unvegetated bed instead had a cumulative
CO$_2$(eq) emission of 3.81 kg m$^{-2}$.

**Figure 4** – Cumulative CO$_2$(eq) emission trends for the three beds.

Considering the plant biomass C content (Tab.3) and the beds biomass yield (Tab.2), *P. australis* and *A. donax* fixed 11.61 and 27.03 kg CO$_2$(eq) m$^{-2}$ respectively, showing a positive balance, while the unvegetated bed, as expected, had a negative balance (Tab.4). Since CWs are multiyear wastewater depuration systems, where the C fixed in the plants belowground biomass, after settlement phase, remains stable due to the root systems turnover, we can exclude it (Tab.4 column$^{(3)}$) from the CO$_2$(eq) balance. Moreover assuming a yearly aboveground biomass cut agronomy management, the CO$_2$(eq) balance for the two vegetated beds showed that during the trial period (about 9 months) they acted as CO$_2$(eq) sinks, with an atmosphere CO$_2$(eq) uptake equal to 1.30 and 8.35 kg m$^{-2}$ for *P. australis* and *A. donax* respectively (Tab.4). The aboveground biomass could be used to produce renewable energy, in fact estimating the higher heating values (HHV) using the C fixed in the biomass, in accordance with Demirbas (1997) formula $\text{HHV} = 0.196 \times \%C + 14.119$. *A. donax* and *P. australis* have an HHV respectively of 22.96 and 22.51 MJ kg$^{-1}$.  

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Table 3 – Percentage carbon content in biomass fractions

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (%C)</th>
<th>Belowground biomass (%C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Roots 0-20 cm</td>
</tr>
<tr>
<td>P. australis</td>
<td>42.83</td>
<td>34.31</td>
</tr>
<tr>
<td>A. donax</td>
<td>45.09</td>
<td>35.89</td>
</tr>
</tbody>
</table>

Table 4 – Beds CO$_2$(eq) balance (kg m$^{-2}$)

<table>
<thead>
<tr>
<th>Species</th>
<th>CO$_2$(eq) emitted$^{(1)}$</th>
<th>CO$_2$(eq) fixed total biomass$^{(2)}$</th>
<th>CO$_2$(eq) fixed aboveground biomass$^{(3)}$</th>
<th>CO$_2$(eq) total balance$^{(2-1)}$</th>
<th>CO$_2$(eq) partial balance$^{(3-1)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. australis</td>
<td>7.34</td>
<td>11.61</td>
<td>8.64</td>
<td>4.27</td>
<td>1.30</td>
</tr>
<tr>
<td>A. donax</td>
<td>8.07</td>
<td>27.03</td>
<td>16.42</td>
<td>18.96</td>
<td>8.35</td>
</tr>
<tr>
<td>Unvegetated</td>
<td>3.81</td>
<td>------</td>
<td>------</td>
<td>-3.81</td>
<td>-3.81</td>
</tr>
</tbody>
</table>

The C3 plants studied, under Mediterranean environment, determine a positive CO$_2$(eq) balance. A. donax that has a high photosynthetic rate and productivity similar to those of C4 species (Christou et al., 2001), fixing more than six times CO$_2$(eq) in its aboveground biomass than P. australis. Considering that the depuration efficiency, in terms of wastewater pollutant abatement, is not significantly different between the two plant species (Idris et al., 2012a), A. donax could be used in CW in Mediterranean environment. Nevertheless, A. donax is a perennial plant that produces high quantity of rhizomes concentrated in bed first layer, that could result after years of operation in a possible decrease of its efficiency and / or an increase in maintenance costs. Although the result showed interesting prospective for A. donax, to date, only few studies are carried out on this plant and in small experimental CW beds (Calheiros et al., 2010, 2012; Idris et al., 2012a,b); therefore long terms study are needed to validate the effects of this species on CW depuration efficiency and GHGs emission prior to give information for technology transfer.

**Conclusions**

CWs are natural-like systems widely used to treat different wastewaters where depuration processes determine greenhouse gases emission. With this in mind Søvik et al. (2006) reported that the question then arises if CWs, used to protect freshwater ecosystems, are a solution to an environmental problem or if they substitute one problem with another, reducing water pollution but increasing greenhouse gases emission.
The results achieved in the present paper confirm the role of plants in CO₂ and CH₄ emissions from CWs that for CO₂ determine higher significantly emission from vegetated beds than unvegetated one. Nevertheless to have a more complete view to answer the question posed by Søvik et al. (2006) the balance of CO₂(eq) has to be considered. Both the vegetated beds showed a positive balance (CO₂ sink) whereas the unvegetated one a negative value (CO₂ source) confirming that vegetation in CWs contributes to enhance the environmental value of this system of wastewater depuration. Although A. donax fixed in the aboveground biomass 1.9 more times CO₂(eq) than P. australis it had a positive balance of CO₂(eq) 6.4 times greater than P. australis. These positive preliminary results encourage further studies to confirm the A. donax promising role as vegetation in Mediterranean CWs.
Chapter V

CO₂ and CH₄ emissions from horizontal subsurface Mediterranean constructed wetlands vegetated with different plant species
Abstract

The aim of this research was to evaluate the role of constructed wetland (CW) horizontal sub-surface flow pilot-plant beds vegetation, comparing five perennial herbaceous plant species with an unvegetated bed, on carbon dioxide (CO₂) and methane (CH₄) emissions, and CO₂(eq) budgets. The research was conducted from April 1st to November 30th in 2012 and 2013 in a pilot plant located in San Michele di Ganzaria (Sicily, Italy) that treated urban wastewaters, studying Phragmites australis (Cav.) Trin. ex Steud. (common reed), Arundo donax L. (giant reed), Cyperus papyrus L. (papyrus), Chrysopogon zizanioides (L.) Roberty (vetiver) and Miscanthus x giganteus Greef et Deu. (mischantus).

Results showed a greater aboveground biomass yield in the second experimental year than the first one for all species except vetiver, which showed a 10.5% reduction. Considering CWs gases emission, a significantly higher CO₂ emission was monitored in 2013 than 2012 whereas CH₄ had the opposite trend. Emission of both gases was influenced by season and plant species. The lower CO₂ emission (median value 4.7 g m⁻² d⁻¹) was monitored during the spring seasons; no significantly different CO₂ emission was found between summer and autumn. An opposite trend was observed for CH₄, with higher emission (median value 0.153 g m⁻² d⁻¹) during spring and lower during autumn (0.004 g m⁻² d⁻¹). A. donax, M. giganteus and P. australis determined significantly higher CO₂ emission than C. papyrus, C. zizanioides and unvegetated bed.

At the end of the two years trial period vegetated beds showed a positive CO₂(eq) balance whereas, as expected, it was negative for the unvegetated bed, with a cumulative CO₂(eq) emission of 5.53 kg m⁻². The best results were calculated for A. donax that showed the highest values in both CO₂(eq) total balance (40.52 kg CO₂(eq) m⁻²) and CO₂(eq) partial balance (21.39 kg CO₂(eq) m⁻²). Obtained results confirm the active and central role of plant species used in the CW systems.

Introduction

Constructed wetlands (CWs) are natural-like systems widely used for wastewater treatment (Barbera et al., 2009; Vymazal, 2013; Tamiazzo et al., 2015). CWs carbon (C) cycles contribute to the global greenhouse gases (GHGs) balance through their carbon dioxide
(CO₂) and methane (CH₄) emissions. In particular they can act as CO₂ sinks by photosynthetic CO₂ assimilation from the atmosphere (Maucieri et al., 2014a) or as a source of CO₂ through bed respiration (Barbera et al., 2014a) and/or organic matter fermentation (CH₄) (Brix et al., 2001). Søvik et al. (2006) reported that the question then arises if CWs, used to protect freshwater ecosystems, are a solution to an environmental problem or if they replace one problem with another by reducing water pollution, yet increasing GHGs emission. Pan et al. (2011), in an estimated life-cycle GHGs emission study concluded that a vertical subsurface flow CW emitted only about 50% of CO₂(eq) with respect to a conventional system to remove 1 kg of BOD. Mitsch et al. (2013) showed that most wetlands are net C sinks providing many ecosystem services in addition to C sequestration; also considering the savings that wetlands give us from fossil fuel consumption for the ecosystem services (e.g. water quality improvement) their service as carbon sinks is even greater.

CWs are increasingly widespread for wastewater treatment in small communities and households where, in addition to the fundamental purifying function, they also have a decorative function that imposes the choice of plants with high functional, amenity, and aesthetic values (Ghermandi et al., 2010). Several studies confirm the role of vascular plants in CWs on GHGs flux by their presence, phenology, density and species composition (Segers, 1998; Ström et al., 2005; Liikanen et al., 2006; Picek et al., 2007; Wang et al. 2008b; Maltais-Landry et al. 2009a; Maucieri et al., 2014b; Barbera et al., 2014b). In fact plant species with different anatomy and physiology and so different oxygen (Wigand et al., 1997) and exudate release levels (Ström et al., 2003) determine different CO₂:CH₄ ratio emission and global warming potential (GWP), given that CH₄ has a 25 times higher effect on GWP than CO₂ (IPCC, 2007). Therefore the choice of the best plant species, besides aesthetic value, which is mainly subjective, has to combine high removal efficiency with low environmental impact.

With this in mind, the aim of this research was to study the carbon dioxide (CO₂) and methane (CH₄) emissions and CO₂(eq) budgets of CW horizontal sub-surface flow pilot-plant beds vegetated with five perennial herbaceous plant species compared with an unvegetated bed.
Materials and Methods

Study site

The research was conducted from April 1st to November 30th in 2012 and 2013 in a pilot plant located in San Michele di Ganzaria (Eastern Sicily – latitude 37°30’ North, longitude 14°25’ East, altitude 370 m a.s.l.). The area has a typical Mediterranean climate (Köppen climate classification), with medium rainfall (approximately 500 mm y⁻¹) mainly in the winter. The pilot plant consisted of six parallel horizontal subsurface flow (HSSF) beds; five vegetated with Phragmites australis (Cav.) Trin. ex Steud. (common reed), Arundo donax L. (giant reed), Cyperus papyrus L. (papyrus), Chrysopogon zizanioides (L.) Roberty (vetiver) or Miscanthus x giganteus Greef et Deu. (miscanthus) and the last unvegetated. Each bed is rectangular in shape with a surface area of 4.5 m² (1.5 m × 3.0 m) and was built of concrete, partially buried, and lined with an impermeable membrane. The beds were filled, to an average depth of 0.6 m, with 10–15 mm volcanic gravel; during the monitoring period the water depth was about 0.55 m. Wastewater inflow was 40 L h⁻¹ with a hydraulic retention time of about 22 hours. Beds were used as tertiary treatment of the urban effluent from a conventional wastewater treatment plant (trickling filter). During summer 2012 was added a sedimentation tank was added between the conventional plant and HSSF beds. Vegetated beds were planted in December 2011 at a density of 5.5 plants m⁻² except for C. papyrus that for logistical reasons, was planted in June 2012 with a relative influence on data magnitude.

Meteorological variables

A CR510 automatic weather station (Campbell Scientific, Logan, UT) was installed close to the experimental plant to measure rainfall, air temperature, wind speed and global radiation.

Gas sampling and analyses

CO₂ and CH₄ sampling and analysis, in both years, were performed from April 1st (vegetative regrowth) to November 30th (end of vegetative cycle). The gas sampling did not cover growing plants.
CO₂ emissions were estimated *in situ* using the static-stationary chamber technique. The cylindrical chambers, of PVC, were 35 cm in height and 12.5 cm wide. The bottom part (20 cm) was permanently inserted in the gravel substrate and the chamber was sealed with a lid in which the CO₂ emitted from the bed was absorbed in a sodium hydroxide (NaOH) solution trap using enhancements reported in Barbera et al. (2014a) to reduce experimental error (Jensen et al., 1996). The CO₂ traps, two in each bed, were replaced every ten days so the monthly total beds respiration (respiration of bed microbes, roots and rhizomes) was calculated based on a decadal dataset.

CH₄ flux was measured using the static non-stationary chamber technique (Barbera et al., 2014b) three times a month in two microsites in order to replicate the measures. The flux cylindrical chamber, 42 cm high and 20 cm wide, was inserted into the gravel substrate using a permanent ring inserted into substrate before the beginning of measurements to prevent soil disturbance in each site. The surface CH₄ flow was determined by measuring the temporal change in CH₄ concentration inside the chamber using a portable FID (Crowcon Gas-Tec®) detecting CH₄ concentrations down to parts per million levels.

**Biomass sampling and analyses**

In December 2012 and 2013 aboveground and belowground biomass were sampled from three sites in each bed. The belowground biomass was collected at three depths (0-20, 20-40 and 40-60 cm) and was divided in roots and rhizomes. Biomass subsamples were homogenized for quality analysis: biomass dry weight was determined using a thermo-ventilated oven at 65 °C until constant weight was reached. Biomass C content was calculated using the average percentage C content in the aerial part, roots and rhizomes dry biomass reported in Maucieri et al. (2014b) and Barbera et al. (2014b) (Tab.1).

**Table 1** – Percentage carbon content in biomass fractions

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (% C)</th>
<th>Belowground biomass (% C)</th>
<th>Roots</th>
<th>Rhizomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. donax</em></td>
<td>45.09</td>
<td></td>
<td>38.18</td>
<td>42.81</td>
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<tr>
<td><em>M. giganteus</em></td>
<td>45.08</td>
<td></td>
<td>40.74</td>
<td>43.29</td>
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<tr>
<td><em>P. australis</em></td>
<td>42.83</td>
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<td>36.42</td>
<td>40.19</td>
</tr>
<tr>
<td><em>C. zizanioides</em></td>
<td>42.49</td>
<td></td>
<td>38.68</td>
<td>-------</td>
</tr>
<tr>
<td><em>C. papyrus</em></td>
<td>37.05</td>
<td></td>
<td>39.42</td>
<td>-------</td>
</tr>
</tbody>
</table>
**Growing season CO$_2$ (eq) balance**

Carbon environmental balance was calculated considering the CO$_2$ and CH$_4$ emissions and the storage of fixed carbon in plant biomass in terms of CO$_2$ (eq) using the following equation:

$$\text{CO}_2\text{(eq)} = C_{\text{biomass}} \times \left(\frac{44}{12}\right) - \text{CO}_2 - (\text{CH}_4 \times 25)$$

where CO$_2$ and CH$_4$ were the GHGs emission measured during the growing season; CH$_4$ emission for CO$_2$ (eq) budgets was computed as 25 times CO$_2$ (IPCC, 2007); the value 44 represents the molecular weight of CO$_2$, and 12 is the C atomic weight. According to Mander et al. (2008) the aboveground plant biomass respiration was not considered, assuming that respired C was previously assimilated by plant gross photosynthesis.

**Statistical analysis**

The normality of data was tested using the Kolmogorov–Smirnov, Lilliefors, and Shapiro–Wilk tests. CO$_2$ and CH$_4$ emissions from the study sites didn’t show normal distribution, so the Kruskal–Wallis and Mann-Whitney non-parametric tests were applied to check the significance of differences (accepted at the level of p<0.05) using combination of replication in time and space.

**Results and Discussion**

**Environmental parameters**

Meteorological data recorded at the site during the monitoring periods are reported in figure 1. Cumulative rainfall from April to November was 102.8 mm and 137.0 mm respectively in 2012 and 2013, the air temperature, in both years, reached maximum value in July and minimum in November. The average solar radiation values in 2012 and 2013 were 21.5 MJ m$^{-2}$ d$^{-1}$ and 21.3 MJ m$^{-2}$ d$^{-1}$ respectively. Average wind speed was generally below 1 m s$^{-1}$. 
Plants biomass production

At the end of the first growing season (December 2012) only plants in the sample points were harvested whereas the remaining aerial biomass was harvested in February 2013. Plants’ regrowth response was not influenced by harvest period except for papyrus that did not regrow when it was cut in December. At the end of the second growing season, *A. donax* showed the highest total plant biomass yield (159.58 Mg ha\(^{-1}\)) followed by *M. giganteus* (110.38 Mg ha\(^{-1}\)), *P. australis* (81.28 Mg ha\(^{-1}\)), *C. papyrus* (56.49 Mg ha\(^{-1}\))
and *C. zizanioides* (48.30 Mg ha\(^{-1}\)). Considering the plant biomass fractions, during the second year, giant reed showed the highest rhizomes production (53.91 Mg ha\(^{-1}\)) whereas papyrus the highest roots production (20.55 Mg ha\(^{-1}\)). Aboveground biomass increased in the second experimental year in all species except for vetiver, which had 10.5% less biomass (Tab.2). *A. donax* showed the highest aboveground biomass confirming the results obtained by Borin et al. (2013a) under high water and nutrient availability. Roots density declined with depth in the bed vegetated with *M. giganteus* and *C. papyrus* whereas in those vegetated with *A. donax* and *P. australis* higher density was found in the 20-40 cm gravel layer. *C. zizanioides* only produced roots in the first 20 cm layer confirming the results reported in the same system by Barbera et al. (2014b). De Stefani et al. (2011), using vetiver plants in floating elements, reported not very successful results for this species, because its establishment was slow and the average root depth was 20 cm 1 month after installation with modest root growth in the following 5 months. Rhizomes were distributed only in the first 20 cm of substrate for *A. donax* and in the 0-40 cm layer for *P. australis* and *M. giganteus* (Tab.2). At the end of the study period *C. zizanioides* showed the highest aboveground:belowground ratio (12.85) whereas the lowest was calculated for *M. giganteus* (1.45; Tab.2).
Table 2 – Aboveground and belowground biomass production at the end of the 2013 study period and plants fraction incidence (% increase or decrease compared to 2012 study period)

<table>
<thead>
<tr>
<th>Species</th>
<th>Aboveground biomass (Mg ha(^{-1}))</th>
<th>ROOTS</th>
<th>ROOTS</th>
<th>ROOTS</th>
<th>RHIZOMES</th>
<th>RHIZOMES</th>
<th>RHIZOMES</th>
<th>RHIZOMES</th>
<th>Roots: Rhizomes ratio</th>
<th>Aboveground:Belowground ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. donax</td>
<td>105.38 ± 27.94 (+3.6%)</td>
<td>2.03 ± 0.21 (-60.5%)</td>
<td>3.09 ± 0.27 (+35.0%)</td>
<td>1.01 ± 0.33 (+43.6%)</td>
<td>116.31 ± 2.89 (+46.3%)</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>0.01</td>
<td>1.94</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>65.35 ± 8.24 (+38.5%)</td>
<td>12.63 ± 0.91 (+94.9%)</td>
<td>3.82 ± 0.48 (+86.9%)</td>
<td>0.58 ± 0.16 (+100%)</td>
<td>56.99 ± 3.97 (+43.0%)</td>
<td>4.65 ± 0.93 (+100%)</td>
<td>-------</td>
<td>0.55</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>P. australis</td>
<td>64.35 ± 3.43 (+13.7%)</td>
<td>0.16 ± 0.04 (-51.8%)</td>
<td>2.65 ± 0.43 (+70.6%)</td>
<td>0.38 ± 0.06 (+68.4%)</td>
<td>16.55 ± 1.02 (+22.1%)</td>
<td>17.23 ± 0.76 (+65.1%)</td>
<td>-------</td>
<td>0.14</td>
<td>3.80</td>
<td></td>
</tr>
<tr>
<td>C. zizanioides</td>
<td>44.81 ± 1.03 (-10.5%)</td>
<td>6.67 ± 0.41 (+53.5%)</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>12.85</td>
</tr>
<tr>
<td>C. papyrus</td>
<td>35.94 ± 3.54 (+69.3%)</td>
<td>16.03 ± 0.35 (+91.0%)</td>
<td>6.37 ± 0.56 (+93.7%)</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>1.75</td>
<td></td>
</tr>
</tbody>
</table>
**GHGs emission**

A significantly higher CO$_2$ emission was monitored in 2013 than 2012 (Fig.2) with median values of 10.6 g m$^{-2}$ d$^{-1}$ and 7.0 g m$^{-2}$ d$^{-1}$ respectively. Tanner et al. (1998) reported a substantial organic matter accumulation in constructed gravel-bed wetlands over a five-year period with a gradual increase due to wastewater loadings. Mander et al. (2008), calculating C balance in a HSSF CW, found an annual C sequestration of 1.5–2.2 kg C m$^{-2}$ incorporated in phytomass and/or substrate of the wetland system that acts as a strong carbon sink. In our study the higher CO$_2$ emission during the second year can be due to organic matter accumulation determined by gravel filtration effect in all beds, and to both higher aboveground and belowground biomass production in vegetated ones (Tab.2). In our research vegetated beds CO$_2$ emission was positively correlated (p<0.05) with both aboveground ($r^2$ 0.78) and total plant biomass ($r^2$ 0.71). CO$_2$ fluxes increasing with higher plant biomass (Liikanen et al., 2006) may be a function of intensified bacterial activity (Gagnon et al., 2007) because of more labile C fractions accessible via plant exudates (Zemanová et al., 2010), which represented up to 20% of aboveground biomass production (Picek et al., 2007).

![Box-plot diagram of beds carbon dioxide emission in different years.](image)

**Figure 2** – Box-plot diagram of beds carbon dioxide emission in different years. Different letters indicate significant differences at p < 0.05 by Mann-Whitney test.
On the average of years and monitored beds, a significantly lower emission was detected during the spring (median value 4.7 g m\(^{-2}\) d\(^{-1}\)), instead no significantly different emission was found between summer and autumn with a median value of 10.9 g m\(^{-2}\) d\(^{-1}\) (Fig.3). The higher summer emissions were due to the higher average air temperature and the plants full development and activity. Temperature is one of the most important factors regulating the rate of microbial processes, positively influencing CO\(_2\) production when temperatures rise (Liikanen et al., 2006; Stadmark and Leonardson, 2005, 2007). The high autumn emission was probably due to the higher COD and BOD\(_3\) wastewater content (data not shown).

**Figure 3** – Box-plot diagram of beds carbon dioxide emission in different seasons. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

Plant species also significantly influence bed respiration with significantly higher CO\(_2\) emissions measured in the beds vegetated with *A. donax*, *M. giganteus* and *P. australis* and lower ones in those vegetated with *C. papyrus* and *C. zizanioides* and unvegetated bed (Fig.4). Although CO\(_2\) is a major GHG, a limited number of studies have focussed on CO\(_2\) emissions in CWs (Mander et al., 2014a) and only a few of them compared plant presence and species effects. In previous studies, the significant roles played by vegetation presence (Ström et al., 2007) and plant species used (Verville et al., 1998; Maltais-Landry et al., 2009a; Barbera et al., 2014b), on CO\(_2\) emission have been shown, with higher CO\(_2\) fluxes in planted vs. unplanted CWs (Maucieri et al., 2014b; Maltais-
Landry et al., 2009a). Our results showed that it seems to be true only for the plants that have a high biomass production (Fig.4 and Tab.2); in fact, as previously reported, no different CO$_2$ emission was monitored among the beds vegetated with *C. papyrus* and *C. zizanioides* and unvegetated one.

![Figure 4 – Box-plot diagram of beds carbon dioxide emission. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

In our study the higher average bed CO$_2$ emission, considering the two monitored periods, was found with *A. donax* (17.6 ± 7.0 g CO$_2$ m$^{-2}$ d$^{-1}$) followed by *P. australis* (16.0 ± 6.8 g CO$_2$ m$^{-2}$ d$^{-1}$), *M. giganteus* (15.7 ± 8.7 g CO$_2$ m$^{-2}$ d$^{-1}$), *C. zizanioides* (6.7 ± 3.7 g CO$_2$ m$^{-2}$ d$^{-1}$), *C. papyrus* (4.9 ± 2.7 g CO$_2$ m$^{-2}$ d$^{-1}$) and unvegetated bed (4.8 ± 3.7 g CO$_2$ m$^{-2}$ d$^{-1}$). Søvik et al. (2006), in a comparative study on GHGs emission from HSSF CWs sites in Northern Europe (Estonia, Norway and Poland), reported an average CO$_2$ emission ranging from 2.9 ± 0.6 to 10.6 ± 0.6 g m$^{-2}$ d$^{-1}$ during summer season (May - early November). Picek et al. (2007), in an HSSF CW bed vegetated with *P. australis* that treated combined sewage and stormwater runoff, reported CO$_2$ emissions varying between 27.2 g m$^{-2}$ d$^{-1}$ (end of June) and less than 4.8 g m$^{-2}$ d$^{-1}$ (toward the end of the growing season).

Biennial cumulative beds respiration differed among the beds, the highest value was calculated for the bed vegetated with *A. donax* (8.6 kg CO$_2$ m$^{-2}$); *P. australis* and *M. giganteus* showed similar values with 7.8 kg CO$_2$ m$^{-2}$ and 7.7 kg CO$_2$ m$^{-2}$ respectively.
The lowest cumulative emissions were monitored in the beds vegetated with *C. zizanioides* (3.2 kg CO$_2$ m$^{-2}$) and *C. papyrus* (2.0 kg CO$_2$ m$^{-2}$) and unvegetated bed (2.3 kg CO$_2$ m$^{-2}$). Vegetated beds respiration includes both autotrophic and heterotrophic respiration whereas unvegetated bed only heterotrophic one. This means that heterotrophic respiration fraction compared with total bed respiration represented 29.9%, 29.5%, 26.7% and 71.9% in the beds vegetated with *M. giganteus*, *P. australis*, *A. donax* and *C. zizanioides* respectively.

The CH$_4$ fluxes were significantly higher in the first studied year (Fig.5).

![Figure 5](image)

**Figure 5** – Box-plot diagram of beds methane emission in different years. Different letters indicate significant differences at $p < 0.05$ by Mann-Whitney test.

On the average of years and beds, season significantly influenced methane emissions (Fig.6) with the highest median value measured during the spring (0.153 g m$^{-2}$ d$^{-1}$), followed by summer (0.098 g m$^{-2}$ d$^{-1}$) and autumn (0.004 g m$^{-2}$ d$^{-1}$). The higher CH$_4$ emission during the first operating year was due to the high CH$_4$ emissions during the first spring season. These high emissions were probably due to the low inlet wastewater quality during the first CWs operating season, which had a higher organic suspended solid content and, as reported in Maucieri et al. (2014b) in vegetated beds also probably due to the plants settlement phase characterized by: 1) a fast root system development resulting in high exudates release that improved methanogen microorganisms activities (Brix et al., 2001; Ström et al., 2003; Saarnio et al., 2004); 2) the incomplete root system
development that determined less oxygen presence in the bed and so lower CH\textsubscript{4} oxidation rate. Furthermore Stadmark and Leonardson (2007) reported more CH\textsubscript{4} production by sediments of constructed ponds in spring than in summer, when incubated at identical temperature. Given that the proportion of organic matter in the sediment did not differ between the two seasons the authors suggested that these results demonstrate that the quality of the sediment as substrate for heterotrophic bacteria and methanogens varies between seasons, and that this affects the amount and composition of greenhouse gases produced.

![Box-plot diagram of beds methane emission in different seasons. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

**Figure 6** – Box-plot diagram of beds methane emission in different seasons. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

Field experiments (Søvik et al., 2006) showed that CH\textsubscript{4} emissions from CWs vary over the year primarily due to temperature. Tanner et al. (1997) found a correlation between air temperature and CH\textsubscript{4} emissions, and Sorrel et al. (1997) reported significantly lower methanogenesis at 12 °C than 30 °C. Zhu et al. (2007) found that under low temperature the substrates for methanogenesis are reduced and consequently CH\textsubscript{4} emission drops. On the other hand, during the second experimental spring maintenance was needed on input and output beds pipelines, determining a break of the wastewater flow for about two months. In this period the plants were kept in the CW systems with zero discharge and replacing the evapotranspiration rate with freshwater every three days. The water table fluctuation inside the beds and the input of freshwater, enhancing oxic condition and
reducing degradable organic matter input, negatively affected the methanogenic bacteria, reducing CH$_4$ emissions. In fact the CH$_4$ flux from CWs is dependent on the quantitative variation of methanogenic and methanotrophic bacteria populations (Truu et al., 2009), which can be positively affected by organic matter availability and negatively affected by water level fluctuation (Mander et al., 2014). Indeed methanogenic communities are more sensitive to changes in sediment redox status than methanotrophs (Altor and Mitsch, 2006) with a slower recovery of metabolic activity in the former than the latter once optimum conditions for their metabolism are restored (Whalen and Reeburg, 2000).

Considering beds, significantly higher CH$_4$ emission was detected in the bed vegetated with $M$. giganteus and unvegetated bed than the one vegetated with $C$. papyrus (Fig.7). Although papyrus CH$_4$ emission data showed an interesting result, it should be considered with caution due to the different transplanting period (summer 2012) than other species. Maltais-Landry et al. (2009a) reported higher CH$_4$ fluxes from unplanted HSSF CWs units than planted ones, confirming the species-specific effect. Macrophytes influencing CWs microbial processes can modify (increase or reduce) CH$_4$ emissions by the balance of CH$_4$ formation and oxidation (Wang et al., 2008b; Wang et al., 2013).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig7.png}
\caption{Box-plot diagram of beds methane emission. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.}
\end{figure}

In our biennial study cumulative CH$_4$ emissions, from April to November, differed among the beds; the highest value was calculated for the bed vegetated with $M$. giganteus
(295.2 g m⁻²) followed by C. zizanioides (225.8 g m⁻²), A. donax (170.3 g m⁻²), P. australis (150.4 kg m⁻²), unvegetated bed (127.8 g m⁻²) and C. papyrus (17.8 g m⁻²). The higher methane emissions from vegetated beds can be attributed to more labile carbon being accessible via plant exudates (Zemanová et al., 2010). In fact CH₄ emission rates and the potential CH₄ production are dependent on substrate quality and the linkage between root exudation of labile carbon, e.g. acetate (Ström et al., 2003).

**Growing season CO₂(eq) balance**

At the end of the two years vegetated beds showed different positive CO₂(eq) balances presenting a similar wastewater depuration efficiency (Toscano et al., 2015). As expected a negative CO₂(eq) balance (Tab.3) and lower depuration efficiency were found for the unvegetated bed (Toscano et al., 2015) with a cumulative CO₂(eq) emission of 5.53 kg m⁻². C. papyrus showed the lowest CO₂(eq) emission, although this result is undoubtedly interesting, as previously described, caution should be used due to the postponed transplanting date and not homogenous regrowth during the second year.

**Table 3 – Beds CO₂(eq) balance (kg m⁻²)**

<table>
<thead>
<tr>
<th>Species</th>
<th>CO₂(eq) emitted⁽¹⁾</th>
<th>CO₂(eq) fixed total biomass⁽²⁾</th>
<th>CO₂(eq) fixed aboveground biomass⁽³⁾</th>
<th>CO₂(eq) total balance (2-1)</th>
<th>CO₂(eq) partial balance⁽³⁾⁻⁽¹⁾</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. donax</td>
<td>12.83</td>
<td>53.35</td>
<td>34.22</td>
<td>40.52</td>
<td>21.39</td>
</tr>
<tr>
<td>M. giganteus</td>
<td>15.04</td>
<td>29.69</td>
<td>17.44</td>
<td>14.65</td>
<td>2.40</td>
</tr>
<tr>
<td>P. australis</td>
<td>11.56</td>
<td>24.23</td>
<td>18.83</td>
<td>12.67</td>
<td>7.27</td>
</tr>
<tr>
<td>C. zizanioides</td>
<td>8.89</td>
<td>15.64</td>
<td>14.69</td>
<td>6.75</td>
<td>5.80</td>
</tr>
<tr>
<td>C. papyrus</td>
<td>2.44</td>
<td>10.26</td>
<td>7.01</td>
<td>7.81</td>
<td>4.57</td>
</tr>
<tr>
<td>Unvegetated</td>
<td>5.53</td>
<td>------</td>
<td>------</td>
<td>-5.53</td>
<td>-5.53</td>
</tr>
</tbody>
</table>

Considering the plant biomass C content (Tab.1) and the beds biomass yield (Tab.2) the best results were obtained for A. donax that showed the highest values in both CO₂(eq) total balance (40.52 kg CO₂(eq) m⁻²) and CO₂(eq) partial balance (21.39 kg CO₂(eq) m⁻²) (Tab.3). M. giganteus showed the second-best results for CO₂(eq) total balance, but the worst CO₂(eq) partial balance among vegetated beds. The bed vegetated with C. zizanioides showed the lowest CO₂(eq) total balance (6.75 kg CO₂(eq) m⁻²).

All the plants studied, under Mediterranean environment, determine a positive CO₂(eq) balance in the CW pilot plant. CWs are multiyear wastewater depuration systems, where the C fixed in the plants belowground biomass, after settlement phase, remains stable.
due to the root systems turnover, therefore the CO$_{2\text{(eq)}}$ partial balances have to be considered for a long evaluation period. With this in mind A. donax seems to be the best environmentally friendly species to use in the studied climate condition, followed by P. australis.

**Conclusions**

Aboveground biomass yield increased in the second experimental year for all species except for vetiver for which a 10.5% reduction of the biomass produced was detected after the first year cutting. During the second growing season A. donax showed the highest aboveground biomass (105.38 Mg ha$^{-1}$) and rhizomes (53.91 Mg ha$^{-1}$) production whereas papyrus the highest roots production (20.55 Mg ha$^{-1}$).

Concerning the two GHGs gases fluxes, a significantly higher CO$_2$ emission was monitored in 2013 than 2012 whereas CH$_4$ had an opposite trend. GHGs emission was influenced by season with the lower and higher emissions during spring for CO$_2$ (median value 4.7 g m$^{-2}$ d$^{-1}$) and CH$_4$ (median value 0.153 g m$^{-2}$ d$^{-1}$) respectively. No significantly different CO$_2$ emission was found between summer and autumn. The lowest CH$_4$ emission was monitored during autumn (0.004 g m$^{-2}$ d$^{-1}$). Plant species significantly influenced bed CO$_2$ emission with the higher significant values detected in the beds vegetated with A. donax, M. giganteus and P. australis and the lower ones monitored in the beds vegetated with C. papyrus and C. zizanioides and unvegetated bed. Regarding CH$_4$, significantly higher emission was measured in the bed vegetated with M. giganteus and unvegetated bed than the one vegetated with C. papyrus.

At the end of the two years trial period vegetated beds showed positive CO$_{2\text{(eq)}}$ balance whereas, as expected, a negative CO$_{2\text{(eq)}}$ balance was found for the unvegetated bed with a cumulative CO$_{2\text{(eq)}}$ emission of 5.53 kg m$^{-2}$. The best results were calculated for A. donax that showed the highest values in both CO$_{2\text{(eq)}}$ total balance (40.52 kg CO$_{2\text{(eq)}}$ m$^{-2}$) and CO$_{2\text{(eq)}}$ partial balance (21.39 kg CO$_{2\text{(eq)}}$ m$^{-2}$).

Obtained results confirm the active and key role of plant species used in the CW systems, underlining the need for an environmental impact assessment, besides the depuration efficiency evaluation, in order to maximize the CWs beneficial environment effects. A. donax seems to be the most environmentally friendly species to use under Mediterranean climate conditions, followed by P. australis.
Chapter VI
Treatment performance and greenhouse gas emission of a pilot hybrid constructed wetland treating digestate fluid fraction
Abstract

A pilot hybrid constructed wetland (HCW) treating digestate fluid fraction (DFF) in north-east Italy was monitored from summer 2012 to summer 2013 to determine its depuration efficiency in treating COD, total nitrogen (TN), ammonia nitrogen (NH$_4$-N), nitrate nitrogen (NO$_3$-N), total phosphorus (TP) and orthophosphate (PO$_4$-P). The system was composed of two 5 m$^2$ vertical subsurface flow beds (VF), one planted with *Phragmites australis* (Cav.) Trin. and the other with *Arundo donax* L. working in parallel, followed by a horizontal subsurface flow bed (HF) (5 m$^2$) vegetated with *P. australis* followed by three floating treatment wetland basins (FTW) connected in series. CO$_2$, CH$_4$ and N$_2$O emissions were also measured in situ, in subsurface flow line (SSL) beds. The system was fed with diluted DFF with a total daily load of 0.7 m$^3$. Wastewater samples were collected at the inflow (IN) and outflow of each VF, HF and FTW unit.

The HCW inflow diluted digestate COD median value ranged from 4,580 to 6,000 mg L$^{-1}$ with an average areal load reduction of 134.8 g m$^{-2}$ d$^{-1}$ and 13.8 g m$^{-2}$ d$^{-1}$ in the SSL beds and FTW basins, respectively. The TN, NH$_4$-N and NO$_3$–N HCW average areal load reductions were 14.3, 9.3 and 0.6 g m$^{-2}$ d$^{-1}$ in the SSL and 1.0, 0.6 and 0.2 g m$^{-2}$ d$^{-1}$ in the FWT, respectively. Both SSL and FWT determined a significant TP and PO$_4$-P abatement with a percentage mass removal that reached 59.6% for TP and 60.6% for PO$_4$-P. The greater areal load reductions for all parameters were achieved in the SSL than FWT line. Concerning VF beds vegetation, *P. australis* showed a better growth performance than *A. donax* although the two species did not show significant different pollutant abatement values. *A. donax* did not regrow in the second year, determining an increase in CH$_4$ emission. CO$_2$ emissions did not show significant differences between seasons and subsurface flow beds; with a spring CO$_2$ average emission of 4,539.8 mg m$^{-2}$ h$^{-1}$ and in summer 2013 of 4,261.5 mg m$^{-2}$ h$^{-1}$. The N$_2$O-N emission from SSL beds was 1.27% and 0.87% of TN removal and inlet, respectively.

Introduction

Animal wastewater management is one of the central topics in agronomic and environmental systems, especially in European countries (Martinez et al., 2009) where the Nitrate Directive imposes hard restrictions on its use (Council Directive
91/676/EEC). On the traditional farm, manure was considered an essential and cheap source of fertilizer but nowadays, with the evolution in livestock rearing a huge quantity of wastewater is produced, which is difficult to handle. The negative effects of excess spreading of animal wastes on arable land are well-known both for the soil and water (Smith et al., 2000), so it is necessary to create or implement treatments and methods for proper livestock waste disposal to observe the European Directive (Henkens and van Keulen, 2001; Harrington and Scholz, 2010).

In this context anaerobic digestion has been proposed as a waste treatment, producing biogas as integrative source of income. In recent years, Italy has witnessed a proliferation of agricultural biogas plants mainly located in the Po Valley (Carrosio, 2013). The great majority of plants (94.3%) are managed by farmers, using energy crops biomass and/or livestock manure as raw materials (Fabbri et al., 2013).

During anaerobic digestion, due to the mineralization of part of the organic matter contained in the ingestates a considerable quantity of carbon is removed, but the concentrations of nitrogen and phosphorus in the anaerobic digester effluent (digestate), remain high (Harrington and Scholz, 2010). Given the digestate characteristic composition there are two main possible solutions for its management. It can be considered either as a byproduct to use (agronomic use) or wastewater to treat when land is not available or is a limiting factor. In the latter case digestate treatment with constructed wetlands (CWs) could be a good prospect for reducing this residual pollutant load, due to their removal efficiency (Borin et al., 2013b; Vymazal, 2013), low cost of installation and maintenance (Sooknah and Wilkie, 2004) and low environmental impact (Barbera et al., 2014a,b; Maucieri et al., 2014b). Little information is available on CWs performance for digestate treatment. However data reported in the literature (Tab.1) show a higher concentration abatement of COD, nitrogen and phosphorus forms but obtained in mesocosm experiments (plant surface < 3 m$^2$). The interesting depuration performance therefore has to be confirmed in more extensive CW systems.
Table 1 – Constructed wetlands performance in treating digestate from literature

<table>
<thead>
<tr>
<th>Country</th>
<th>CW type</th>
<th>Surface (m²)</th>
<th>Plant species</th>
<th>COD</th>
<th>TKN</th>
<th>N-NH₄</th>
<th>N-NO₃</th>
<th>TP</th>
<th>References</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IN</td>
<td>OUT</td>
<td>A (%)</td>
<td>IN</td>
<td>OUT</td>
<td>A (%)</td>
</tr>
<tr>
<td>Mexico</td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Lemma spp.</em></td>
<td>2189</td>
<td>197</td>
<td>91</td>
<td>200</td>
<td>84</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Lemma spp.</em></td>
<td>2189</td>
<td>153</td>
<td>93</td>
<td>200</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>Florida</td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Lemma spp.</em></td>
<td>2189</td>
<td>197</td>
<td>91</td>
<td>200</td>
<td>48</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Lemma spp.</em></td>
<td>2189</td>
<td>285</td>
<td>87</td>
<td>200</td>
<td>44</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Control/algal</em></td>
<td>2007</td>
<td>698</td>
<td>65.2</td>
<td>257</td>
<td>41.12</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Control/algal</em></td>
<td>1023</td>
<td>350</td>
<td>65.8</td>
<td>164</td>
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<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Eichhornia crassipes</em></td>
<td>1860</td>
<td>483.6</td>
<td>74</td>
<td>227</td>
<td>35</td>
<td>84</td>
</tr>
<tr>
<td></td>
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<td>miniponds</td>
<td><em>Eichhornia crassipes</em></td>
<td>1103</td>
<td>215</td>
<td>81</td>
<td>164</td>
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<td></td>
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<td>miniponds</td>
<td><em>Pistia stratiotes</em></td>
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<td>201</td>
<td>80</td>
<td>162</td>
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<tr>
<td></td>
<td>FWS</td>
<td>miniponds</td>
<td><em>Hydrocotyle umbellata</em></td>
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<td>260</td>
<td>72</td>
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<tr>
<td>Thailand</td>
<td>VF</td>
<td>1.44</td>
<td><em>Typha angustifolia</em></td>
<td>377</td>
<td>135</td>
<td>64</td>
<td>288</td>
<td>127</td>
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<tr>
<td></td>
<td>VF</td>
<td>1.44</td>
<td><em>Cyperus alternifolius</em></td>
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<td>117</td>
<td>13</td>
<td>127</td>
<td>76</td>
<td>41</td>
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<tr>
<td></td>
<td>FWS</td>
<td>0.78</td>
<td><em>Glyceria maxima</em></td>
<td>13.48</td>
<td>0.1</td>
<td>99</td>
<td>98</td>
<td>16</td>
<td>83</td>
</tr>
<tr>
<td>Ireland</td>
<td>FWS</td>
<td>0.78</td>
<td><em>Glyceria maxima</em></td>
<td>10.06</td>
<td>0.93</td>
<td>91</td>
<td>102</td>
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<tr>
<td></td>
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<td><em>Glyceria maxima</em></td>
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<td>0.46</td>
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<td>145</td>
<td>43</td>
<td>70</td>
</tr>
<tr>
<td>Italy</td>
<td>VF+HF</td>
<td>2.97</td>
<td><em>Juncus maritimus,</em> <em>Typha latifolia,</em> <em>Aster tripolium</em></td>
<td>1192</td>
<td>279.8</td>
<td>76.5</td>
<td>120</td>
<td>15.4</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>VF+HF</td>
<td>2.97</td>
<td><em>Typha latifolia,</em> <em>Aster tripolium</em></td>
<td>820</td>
<td>95.1</td>
<td>88.4</td>
<td>130</td>
<td>2.4</td>
<td>98.2</td>
</tr>
</tbody>
</table>

*phosphorus expressed as orthophosphate; ** inoculum density = 27 g m⁻²; *** inoculum density = 27 g m⁻² and harvest frequency = 3 days; **** inoculum density = 45 g m⁻²; ***** inoculum density = 54 g m⁻² and harvest frequency = 3 days; VF=vertical sub-surface flow CW; HF=horizontal sub-surface flow CW; FWS=free water surface CW; A(%)=percentage concentration abatement.
In the CW systems, during organic and nitrogen load abatement processes, gaseous compounds are released into the atmosphere. Among these gases, CO₂, CH₄, and N₂O are the most environmentally dangerous, acting as greenhouse gases (GHGs). Therefore for a more complete evaluation of environmental sustainability of CWs their GHGs emission should be considered. A few studies have been carried out in the Mediterranean Basin (García et al. 2007; García-Lledó et al., 2011; Barbera et al., 2014a,b; Maucieri et al. 2014b) but, to our knowledge, none in CWs that treated digestate.

The aim of this work was to evaluate the depuration performance and GHG emissions in a pilot hybrid constructed wetland (HCW) treating digestate fluid fraction (DFF) in north-east Italy.

**Materials and Methods**

**Site description**

The experimental activities were carried out in Terrassa Padovana, Padova (Eastern Veneto Region, Italy, latitude 45°14'42"00 North, longitude 11°54'13"32 East, altitude 4 m a.s.l.). The climate of this site is sub-humid, with a mean annual rainfall of about 800 mm uniformly distributed throughout the year, with a higher variability from September to November. The mean annual average temperature is about 12.5 °C.

The pilot HCW is installed at a Charolais beef cattle farm provided with an anaerobic digestion plant that can produce 1 MW d⁻¹ electric energy and 576 kW d⁻¹ thermic energy using slurry (4,000 Mg year⁻¹), corn silage (14,000 Mg year⁻¹) and residues from agriculture (4,000 Mg year⁻¹). This system generates 50 m³ d⁻¹ of digestate, which is then mechanically separated. The DFF is stored in three circular section cisterns (25 m in diameter and 3.5 m in height).

The HCW (Fig.1), designed to treat up to 0.7 m³ d⁻¹ of DFF, consists of: 1) a concrete catch basin (0.8 x 0.8 x 0.8 m); 2) a sedimentation basin (basin 1) (5 x 2 x 1 m); 3) an equalization basin (basin 2) (5 x 1 x 1 m); 4) two sub-surface vertical flow beds (VF) (5 x 1 x 1 m, hydraulic retention time (HRT) 1 day) one vegetated with *Arundo donax* L. (VF A) and the other with *Phragmites australis* (Cav.) Trin. (VF P); 5) a manhole receiving the waste water to be treated from the equalization basin and containing two
pumps that load the two VF beds; 6) a sub-surface horizontal flow bed (HF) (5 x 1 x 1 m, HRT 2 days) vegetated with *P. australis* (HF P); 7) a final catch basin (0.8 x 0.8 x 0.8 m) containing a pump that conveys the discharged water back into the storage tank (in 2012). In 2013 the HCW was connected to a line of three floating treatment wetlands (FTW) installed beside the line of VF and HF to improve the treatment potential. This last consists of three separate square basins functioning in series with an average water depth, during the experimental period, of about 0.3 m and surface areas that differ slightly: 27 m$^2$ in basin 1 (FTW 1), 30 m$^2$ in basin 2 (FTW 2) and 33 m$^2$ in basin 3 (FTW 3) with a HRT of about 40 days. In its complete configuration the HCW is hence composed of one line of VF and HF beds (Sub-surface Line, SSL) and one line of three FTW basins (FTW line, FTWL).

**Figure 1** – Hybrid constructed wetland pilot plant layout.

The HCW pilot plant has been built on an artificial embankment with the basins located at different elevations. The VF beds and HF one were vegetated in summer 2010 at a density of 4 plants m$^{-2}$ and were maintained until July 2012 supplying highly diluted DFF to favor plant settlement and homogenous root system substrate colonization. The VF beds were filled from bottom to top with: 1) 15 cm of washed river bed gravel (Ø 5 cm); 2) 50 cm of washed river bed gravel (Ø 1-2 cm); 3) 20 cm of washed river bed sand (Ø 2 mm); 4) 10 cm of washed river bed gravel (Ø 1-2 cm).
The HF bed was filled from inlet to outlet with: 1) 0.5 m of washed river bed gravel (Ø 5 cm); 2) 4 m of washed river bed gravel (Ø 1-2 cm); 3) 0.5 m of washed river bed gravel (Ø 5 cm).

Each basin of the FTW was waterproofed by sheets of polyolefin and completely covered with ethylene vinyl acetate (EVA) Tech-IA® floating elements: 54 elements in B1, 60 in B2 and 66 in B3. The self-floating elements are rectangular (50 x 90 cm) with eight holes each to sustain plants (De Stefani et al., 2011; Mietto et al., 2013). In this FWS, different plant species were transplanted in July 2011 to test their aptitude in treating DFF (Pavan et al., 2014).

A central data logger managed the digestate flow through to the systems. The load was applied from July to November in 2012 and from March to August in 2013 according to the following procedure. Basin 1 was filled with 100 L of DFF, pumped from the stock tank, and 600 L of water from the drainage ditch accumulated in a basin near the plant system. The DFF dilution was necessary due to the extremely high mean values of total nitrogen (TN) (1,678 mg L⁻¹) and electrical conductivity (EC) (22.4 mS cm⁻¹). It hence represented a simulation of the possible effect of a pre-treatment to be applied to the DFF. The diluted digestate flowed through basin 2 by gravity and was then pumped to the next stage (VF beds). The VF beds were loaded alternately every two days. Load was provided during two and half hours, with four loading cycles of 175 L. The bed was kept completely full of digestate for 24 hours, then the discharge cycle was activated and the bed was emptied in one and half hours. According to this management scheme a VF bed alternates 24 hours full (anaerobic conditions), 20 hours empty (aerobic conditions) and 4 hours for load/discharge.

Following the programmed HRT the digestate flowed from VF bed through a valve to the HF bed and came out at the catch basin where a pump sent the liquid back to the storage tank (in 2012). In 2013 after HF bed the diluted DFF was pumped into the first basin (FTW 1) of the FTWL then moved by gravity to the second and third ones (FTW 2 and FTW 3).

**Meteorological variables and water balance**

The following meteorological data were recorded by the weather station at Tribano, about 10 km from the experimental site: rain (mm), max, min and average air temperature
(°C), wind speed (m s\(^{-1}\)), relative humidity (%), and solar radiation (MJ m\(^{-2}\)d\(^{-1}\)).

Potential evapotranspiration (ET0) was calculated using Penman–Monteith method.

HCW water balance (WB) was determined on the average of each monitored period with the follow equation:

\[
WB = DFF_{in} + P - DFF_{out} \quad \text{(Eq. 1)}
\]

Where \(DFF_{in}\) = diluted DFF inflow rate (L d\(^{-1}\)), \(DFF_{out}\) = diluted DFF outflow rate (L d\(^{-1}\)) and \(P = (R * S)\) where \(R = \text{rain (mm d}^{-1}\)) and \(S = \text{SSL or FWTS top surface area (m}^2\))

### Digestate sampling and analysis

The depuration performance were evaluated with four monitoring cycles of about 15 days: summer and autumn 2012, spring and summer 2013. For each sampling day wastewater was taken from outlet of basin 2 (IN), outlet of VF A, outlet of VF P and outlet of HF P in 2012, adding samples from the outlet of each FTW basin (FTW 1, FTW 2 and FTW 3) in 2013. After sampling, wastewater was kept refrigerated and afterwards analyzed in laboratory to determine TN, ammonium nitrogen (NH\(_4\)-N), nitric nitrogen (NO\(_3\)-N), total phosphorus (TP), orthophosphate (PO\(_4\)-P) and chemical oxygen demand (COD) with the spectrophotometer (Spectrophotometer DR2008 Hach-Lange and specific cuvettes test for each parameter). In situ measurements of pH, redox potential (Eh), dissolved oxygen (DO) and electrical conductivity (EC) were also taken with the Hach Lange, HQ40d multi parametric probe and turbidity with a turbid meter (Hanna Instruments HI83414) and expressed in Nephelometric Turbidity Units (NTU).

The evaluation of HCW treatment performance was based on the:

(a) concentration percentage abatement (A), calculated on median concentration values as (Eq. 2):

\[
A\% = \frac{C_{in} - C_{out}}{C_{in}} \times 100
\]

where \(C_{in}\) is inflow concentration (mg L\(^{-1}\)) and \(C_{out}\) is outflow concentration (mg L\(^{-1}\));

(b) removal efficiency (RE) calculated on median concentration values as (Eq.3):

\[
RE\% = \frac{(C_{in} \times V_{in}) - (C_{out} \times V_{out})}{(C_{in} \times V_{in})} \times 100
\]

where \(C_{in}\) is inflow concentration (mg L\(^{-1}\)), \(V_{in}\) is average inflow volume of synthetic wastewater applied (m\(^3\) d\(^{-1}\)) with daily rainfall volume (mm d\(^{-1}\)) included, \(C_{out}\) is outflow concentration (mg L\(^{-1}\)), \(V_{out}\) is outflow volume detected at the outlet of the unit (m\(^3\) d\(^{-1}\)).
(c) areal load reduction (ALR), that expresses the removed pollutants mass per m\(^2\) of HCW and time (g m\(^{-2}\) d\(^{-1}\)).

**Gas sampling and analyses**

The measurement of CO\(_2\), CH\(_4\) and N\(_2\)O was carried out using the static non-stationary chamber technique in three points of each bed in order to replicate the measures in space. The cylindrical flux chamber, 42 cm high and 20 cm wide, was inserted into the gravel substrate using a permanent ring inserted into substrate before the beginning of measurements to prevent substrate disturbance in each site. GHGs emission were detected measuring their temporal concentration change inside the chamber using a portable FID (Crowcon Gas-Tec®) for CH\(_4\) and infrared sensor for N\(_2\)O (Geotech G200) and CO\(_2\) (Delta OHM HD21AB17). GHGs concentrations were detected down to parts per million levels and fluxes were calculated as reported in Barbera et al. (2014b).

GHGs fluxes were calculated using the following formula (Eq. 4):

\[
\text{GHGs} = \frac{V}{A} \cdot \frac{dc}{dt}
\]

where GHGs flux is expressed in mg m\(^{-2}\) s\(^{-1}\); V (m\(^3\)) is the volume and A (m\(^2\)) the footprint of the flux chamber; ‘c’ is the GHGs concentration (mg m\(^3\)) and ‘t’ the time step (s).

The gas samplings did not cover growing plants and were carried out from 8.00 to 12.00 am on the same days of the 3\(^{rd}\) (spring 2013) and 4\(^{th}\) (summer 2013) depuration performance monitoring cycles. The effect of plant cutting, only on CH\(_4\) emissions, was monitored in autumn 2012 by measuring gas fluxes, for three consecutive days, two weeks before and two weeks after biomass harvest, that was done on October 24\(^{th}\).

**Statistical analysis**

In our experimental design, we use replication through time and space rather than through independent experimental units. The normality of data was checked using the Kolmogorov–Smirnov test. Neither digestate chemical-physical parameters nor GHGs emission showed normal distribution, so the Kruskal–Wallis and Mann–Whitney nonparametric tests were used to check the significance of differences (accepted at the level of p<0.05). Correlations between monitored wastewater parameters were evaluated using Spearman Rank correlation.
**Results and Discussion**

**Meteorological variables and water balance**

Data recorded during the monitoring period (July 2012 – August 2013) (Fig. 2) are typical of a sub-humid area. Cumulative rainfall was 1,197.4 mm, and the average daily air temperature reached 15.2 °C, with its maximum value on July 2\textsuperscript{nd} 2012 and July 7\textsuperscript{th} (28.9 °C) and minimum of -3.5 °C on December 9\textsuperscript{th} 2012. During the same period the average solar radiation that reached the canopy was 14.96 MJ m\textsuperscript{-2} d\textsuperscript{-1}, with the higher monthly average intensity values registered in July (26.34 MJ m\textsuperscript{-2} and 26.22 MJ m\textsuperscript{-2} in 2012 and 2013 respectively) and the lowest in January 2013 (3.22 MJ m\textsuperscript{-2}). Average wind speed was 2.1 m s\textsuperscript{-1}.

**Figure 2** – Temperature, rainfall, solar radiation and wind speed recorded during the study period.
Comparing the monitored days among periods, the highest average daily ET0 was found in summer 2013 (5.4 mm), the lowest in autumn 2012 (1.5 mm); the average daily ET0 in summer 2012 and spring 2013 were 4.6 mm and 3.5 mm, respectively. DFF inflow in SSL was always 700 L d\(^{-1}\) whereas in FWTS it was influenced by SSL evapotranspiration. As expected diluted DFF percentage volume abatement in SSL followed a seasonal trend with the higher values obtained in summer with an average of 55.3\% (Tab.2) and an average in the whole experimental period of 43.4\%.

<table>
<thead>
<tr>
<th>Table 2 – Subsurface flow line water balance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL</td>
</tr>
<tr>
<td>Summer 2012</td>
</tr>
<tr>
<td>DFF(_{in}) (L d(^{-1}))</td>
</tr>
<tr>
<td>P (L d(^{-1}))</td>
</tr>
<tr>
<td>DFF(_{out}) (L d(^{-1}))</td>
</tr>
<tr>
<td>Volume abatement (%)</td>
</tr>
</tbody>
</table>

The great volume abatement, mainly due to beds vegetation, confirms the high evapotranspiration rate of the two species used in this study, in agreement with Borin et al. (2011) and Toscano et al. (2015), underlining the strong effect of vegetation. In 2013, the average volume abatement found in FWTL was 84.5\%. Although this last result is very interesting it must be interpreted with caution considering that was calculated in only two seasons of the same year. In fact Borin et al. (2011) highlighted that ET differs depending on site and throughout the growing season of the same year but also between different years. Therefore, further long-term studies are needed to confirm obtained data.

**Depuration performance**

**On site parameters**

On site measured wastewater parameters during the study period are reported in table 3. Electrical conductivity (EC) median value of the diluted DFF varied from 4.61 (summer 2013) to 5.78 mS cm\(^{-1}\) (spring 2013) with a significant decrease from inlet to outlet both for sub-surface beds and FTW basins in all monitored periods except in summer 2012.

The influent pH ranged from 8.2 to 9.3, and the sub-surface flow beds reduced the wastewater median pH value in all monitored periods by about 0.5 units, with median pH values in SSL outlet ranging from 7.6 (summer 2013) to 8.6 (autumn 2012 and spring 2013). In FTWL outlet pH median values were 8.7 in spring 2013 and 8.4 in summer 2013.
Table 3 – On site measured wastewater parameters (median value)

<table>
<thead>
<tr>
<th></th>
<th>Summer - 2012</th>
<th>Autumn - 2012</th>
<th>Spring - 2013</th>
<th>Summer - 2013</th>
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<tbody>
<tr>
<td></td>
<td>NTU</td>
<td>pH</td>
<td>Eh (mV)</td>
<td>EC (mS cm⁻¹)</td>
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<tr>
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<td>HF P</td>
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<td>8.4 b</td>
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<tr>
<td>SSL % Abatement</td>
<td>19.1</td>
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<tr>
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<td>NTU</td>
<td>pH</td>
<td>Eh (mV)</td>
<td>EC (mS cm⁻¹)</td>
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<tr>
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<td>30.8 b</td>
<td>4.3 b</td>
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<tr>
<td>HF P</td>
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<td>8.6 b</td>
<td>-237.6 b</td>
<td>4.4 b</td>
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<tr>
<td>SSL % Abatement</td>
<td>57.5</td>
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</tr>
<tr>
<td></td>
<td>NTU</td>
<td>pH</td>
<td>Eh (mV)</td>
<td>EC (mS cm⁻¹)</td>
</tr>
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<td>IN</td>
<td>4,466.5 a</td>
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<td>VF A</td>
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<td>FTW 2</td>
<td>545.5 bc</td>
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<td>2.9 bc</td>
</tr>
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<td>FTW 3</td>
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<tr>
<td>SSL % Abatement</td>
<td>58.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTWL % Abatement</td>
<td>93.2</td>
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</tbody>
</table>

The median inflow diluted digestate redox potential significantly decreased after SSF beds in all periods except summer 2013 when no significant difference was shown. No statistical differences were detected for this parameter at the end of FTW system in both spring and summer 2013. As expected, in the first two monitored periods, the significant increase of Eh due to VF beds was in agreement with Vymazal (2010), who reported that anoxic conditions predominated in saturated HF beds while oxic conditions...
prevailed in VF ones. Surprisingly, no clear effects were obtained in 2013 monitored periods, with VF beds outlet Eh values not significantly different than those monitored in the inlet diluted DFF. This is probably due to the organic matter accumulation in the first VF beds substrate layer that reduced oxygen presence in the substrate. Although the HF bed is characterized by more reduction than VF ones, Eh did not significantly change at the wastewater outlet probably due to common reed root system oxygen release (Brix et al., 1992; Brix, 1994).

**COD**

The COD median value of inflow diluted DFF ranged from 4,580 mg L\(^{-1}\) (summer 2012) to 6,000 mg L\(^{-1}\) (spring 2013) (Fig. 3) with a median load of 3.21 kg and 4.20 kg, respectively.

![Figure 3 - Box-plot diagrams of wastewater COD concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

In SSL COD percentage abatement ranged from 21.7\% to 38.6\% in summer and spring 2013, respectively, and from 48.9\% (summer 2013) to 63.3\% (spring 2013) in the
FWTL (Tab.4). VF P bed, in 2012 monitored cycles, showed a significant COD concentration abatement (23.1%) compared with DFF inlet values whereas no significant abatement was found in the VF A outlet. In the two 2013 monitored cycles, DFF COD concentration was not significantly abated in the beds of SSL whereas in the same cycles a significant COD abatement (59%) was observed at the end of FTW 1 compared with inlet values. (Fig.3).

```
Table 4 – HCW pollutant concentration abatement (%)

<table>
<thead>
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<th></th>
<th></th>
<th></th>
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<th></th>
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</thead>
<tbody>
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<td>COD</td>
<td>TN</td>
<td>NH₄⁺-N</td>
<td>NO₃⁻-N</td>
<td>TP</td>
<td>PO₄-P</td>
</tr>
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<td>Summer - 2012</td>
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<td>23.5</td>
<td>40.3</td>
<td>-61.7</td>
<td>12.8</td>
<td>16.6</td>
</tr>
<tr>
<td>Autumn - 2012</td>
<td>22.5</td>
<td>42.9</td>
<td>27.9</td>
<td>-4.9</td>
<td>8.9</td>
<td>10.4</td>
</tr>
<tr>
<td>Spring - 2013</td>
<td>38.6</td>
<td>49.2</td>
<td>53.4</td>
<td>3.1</td>
<td>41.1</td>
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<td>Summer - 2013</td>
<td>21.7</td>
<td>38.5</td>
<td>44.5</td>
<td>21.6</td>
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<tr>
<td>FTWL</td>
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<td></td>
</tr>
<tr>
<td>Spring - 2013</td>
<td>63.3</td>
<td>66.5</td>
<td>63.4</td>
<td>79.8</td>
<td>14.7</td>
<td>13.9</td>
</tr>
<tr>
<td>Summer - 2013</td>
<td>48.9</td>
<td>37.6</td>
<td>19.0</td>
<td>30.4</td>
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```

The higher COD RE was detected in SSL system during the two summer seasons with an average value of about 67% whereas the lower one in autumn 2012 (39.8%). In 2013, the FWT system determined a further COD percentage removal that reached 78.8% in spring and 99.5% in summer at the end of the three FTW basins (Tab.5).

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Table 5 – HCW pollutant removal efficiency (%)

<table>
<thead>
<tr>
<th></th>
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<th></th>
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<td></td>
<td>COD</td>
<td>TN</td>
<td>NH₄⁺-N</td>
<td>NO₃⁻-N</td>
<td>TP</td>
<td>PO₄-P</td>
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<td>66.8</td>
<td>63.8</td>
<td>71.7</td>
<td>23.5</td>
<td>58.8</td>
<td>60.6</td>
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<tr>
<td>Autumn - 2012</td>
<td>39.8</td>
<td>55.6</td>
<td>44.0</td>
<td>18.5</td>
<td>29.2</td>
<td>30.5</td>
</tr>
<tr>
<td>Spring - 2013</td>
<td>57.9</td>
<td>65.1</td>
<td>68.0</td>
<td>33.5</td>
<td>59.6</td>
<td>44.2</td>
</tr>
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<td>Summer - 2013</td>
<td>66.9</td>
<td>74.0</td>
<td>76.6</td>
<td>66.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FTWL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring - 2013</td>
<td>78.8</td>
<td>80.5</td>
<td>78.7</td>
<td>88.2</td>
<td>50.3</td>
<td>49.9</td>
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<tr>
<td>Summer - 2013</td>
<td>99.5</td>
<td>99.4</td>
<td>99.2</td>
<td>99.3</td>
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</tbody>
</table>
```

Considering the COD ALR (Tab.6), the higher values were found in spring 2013 for both SSL (162.1 g m⁻² d⁻¹) and FTWL (15.5 g m⁻² d⁻¹), the lower ones for SSL in autumn 2012 (86.0 g m⁻² d⁻¹) and for FTWL in summer 2013 (12.1 g m⁻² d⁻¹). To our knowledge data available in literature has been obtained in smaller HCW than ours using a lower COD load.
Table 6 – HCW pollutant areal load reduction (g m\(^{-2}\) d\(^{-1}\))

<table>
<thead>
<tr>
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<tr>
<td></td>
<td>COD</td>
<td>TN</td>
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<tr>
<td>Summer 2012</td>
<td>142.7</td>
<td>11.3</td>
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<td>Autumn 2012</td>
<td>86.0</td>
<td>12.0</td>
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<tr>
<td>Spring 2013</td>
<td>162.1</td>
<td>20.0</td>
</tr>
<tr>
<td>Summer 2013</td>
<td>148.2</td>
<td>14.0</td>
</tr>
<tr>
<td>FTWL</td>
<td>COD</td>
<td>TN</td>
</tr>
<tr>
<td>Spring 2013</td>
<td>13.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Summer 2013</td>
<td>10.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Results obtained in this study confirm HCW good performance also under severe load condition. Comino et al. (2013) in a small HCW (2 VF beds + 1 HF bed) that treated different digestate with different pollutant load, found an average COD mass removal of 42.2% and 63.7% respectively with a COD daily load of 59.6 g and 164.0 g. In the same paper, the authors calculated a HCW ALR of 45.5 g m\(^{-2}\) d\(^{-1}\) and 144.9 g m\(^{-2}\) d\(^{-1}\) with lower and higher load, respectively. Sooknaha and Wilkie (2004) using FWS CWs vegetated with floating aquatic macrophytes (water hyacinth or pennywort or water lettuce or a mixture of these species) to treat diluted digestate (1:1 water to digestate; COD = 1013.3 mg L\(^{-1}\)) reported an average abatement of 76%. In our work the DFF average COD concentration in the FWTL inlet was 4,072.2 mg L\(^{-1}\) (about 4 times higher) with an average abatement of 59.4% (only about 0.2 times lower) in the outlet, showing a good performance of FWT with Tech-IA® floating elements under high COD load. During monitoring periods, considering SSL + FTWL, the COD values were negatively correlated with Eh (Spearman R = -0.27; p<0.001), which give us a picture of anoxic conditions, and positively correlated with pH (Spearman R = 0.40; p<0.0001) and NTU (Spearman R = 0.83; p<0.00001). Considering SSF beds, COD values were positively correlated with NH\(_4^+\) diluted digestate content (Spearman R = 0.61; p<0.0001) and NH\(_4^+\)/NO\(_3^−\) ratio (Spearman R = 0.46; p<0.0001), no correlation was found with NO\(_3^−\). Moreover the FTW basins COD values were positively correlated with both NH\(_4^+\) (Spearman R = 0.60; p<0.0001) and NO\(_3^−\) (Spearman R = 0.52; p<0.001), but not with their ratio.

Carrera et al. (2004) in a study to quantify the effect of influent COD/N ratio on industrial high-strength ammonium wastewater biological nitrogen removal process, reported that the nitrification rate was theoretically constant (0.032 g N g VSS\(^{-1}\) d\(^{-1}\)) at COD/N ratios
higher than 4 g COD g N⁻¹ and that a COD/N ratio of 7.1 g COD g N⁻¹ is required to achieve total denitrification. In our experiment COD/N ratio ranged from 10.4 (spring 2014) to 12.2 (summer 2012) in the inlet diluted wastewater and from 9.4 to 14.8 considering all hybrid system beds and basins. These values showed that substrates for denitrifying microorganism populations in digestate were not a limiting factor.

Nitrogen forms

During the experimental periods, TN influent median value concentration ranged from 378.0 mg L⁻¹ (summer 2012) to 657.5 mg L⁻¹ (spring 2013) with significantly lower values at the end of SSL and FTWL in 2012 and 2013, respectively (Fig.4). Considering the diluted DFF TN concentration, during treatment steps, the reduction trend is the same in the two 2013 monitored periods although with higher absolute inlet median values in spring (658 mg L⁻¹) than in summer (404 mg L⁻¹) (Fig.4). This confirms the high buffer effect of HCW system.

Figure 4 – Box-plot diagrams of wastewater TN concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.
The TN abatement achieved by SSL ranged between 23.5% (summer 2012) and 49.2% (spring 2013) and from 37.6% to 66.5% in the FTWL (Tab.4). The RE on the average of monitored cycles was 64.6% in the SSL and 90% in the FTWL, (Tab.5) corresponding to an ALR of 14.3 g m\(^{-2}\) d\(^{-1}\) and 1.1 g m\(^{-2}\) d\(^{-1}\), respectively. Borin et al. (2013b) reported an ARL of 17.5 g m\(^{-2}\) d\(^{-1}\) for a HCW (3 VF beds + 1 HF bed, total surface 130 m\(^{2}\)), that treated pig slurry effluent with daily TN load of 25.8 g m\(^{-2}\). Daily SSL TN load in this study was 20.0 g m\(^{-2}\) therefore considering ARL to TN load per m\(^{-2}\) ratio, the two studies show similar values, 67.8% in Borin et al. (2013b) and 71.5% in this study confirming the high depuration performance of HCW. In fact HCW are primarily used for enhanced removal of TN because different CW flows provide different redox conditions that are suitable for nitrification and denitrification (Vymazal, 2011). HF beds are often anoxic/anaerobic due to permanent saturation of the filtration bed and, hence, provide suitable conditions for denitrification if organics are present; on the other hand, VF beds are aerobic due to intermittent feeding which allows for oxygen diffusion into the filtration bed (Vymazal, 2007). The ammonia nitrogen form represented the major fraction of TN load in the system. Except for spring 2013, when NH\(_4\)-N influent concentrations median value was about 500 mg L\(^{-1}\), in other monitored periods the inlet diluted DFF median value NH\(_4\)-N concentration was slightly lower than 250 mg L\(^{-1}\) (Fig.5). The average SSL NH\(_4\)-N abatement was 41.5% with a lower value monitored in autumn 2012 and higher in summer 2013 (Tab.4). In the 2012 monitoring cycles VF beds did not show significant difference between them. VF beds show significant NH\(_4\)-N abatement compared inlet values only in the autumn whereas HF bed significantly abated NH\(_4\)-N concentration in both summer and autumn (Fig.5).
During 2013, NH$_4$-N concentration abatement followed the same trend as TN (Fig.4 and Fig.5). During the study period, NH$_4$-N RE (Tab.5) was 65.1% and 89.0% in SSL and FWTL, respectively. The highest NH$_4$-N ALR was found, in all monitored cycles, at the end of SSL (Tab.6). In 2013 FWTL further increased NH$_4$-N ARL, with values of 1.0 g m$^{-2}$ d$^{-1}$ and 0.4 g m$^{-2}$ d$^{-1}$ in spring and summer respectively. As already known ammonia removal is pH and temperature dependent (Wallace and Knight, 2006). Reddy et al. (1984) reported that NH$_4$-N volatilization is insignificant if the pH is below 7.5 and, very often, not serious if the pH is below 8.0. At pH 9.3 the ratio between ammonia and ammonium ions is 1:1 and losses via volatilization are significant (Vymazal, 2007). In CW ammonia volatilization process is most significant when the influent water contains high levels of NH$_4$-N and pH exceeds 8.0 (Reddy and D’Angelo, 1997). In our study the digestate pH values, except for SSL in summer 2013, was always higher than 8 (Tab.3) and digestate NH$_4$-N content was high (Fig.5) therefore the N loss by ammonia volatilization was relevant.
Diluted DFF input NO$_3$–N concentration median value ranged between 26.4 mg L$^{-1}$ (summer 2012) and 45.0 mg L$^{-1}$ (spring 2013) (Fig.6). With respect to the inflow the concentration increased along SSL in 2012 (Tab.4), while a significant reduction was measured at the end of the FTWL in 2013 (Fig.6).

Figure 6 – Box-plot diagrams of wastewater NO$_3$–N concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

The SSL NO$_3$–N concentration increase was probably due to more oxygen availability as indirectly confirmed by the positive correlation between digestate NO$_3$–N concentration and Eh (Spearman R = 0.32; p<0.001), in agreement with Vymazal (1995) who reported that nitrification is also influenced by dissolved oxygen. The lowest NO$_3$–N percentage abatement in SSL was observed in autumn when the temperature was lower than other monitored cycles. This is in agreement with Mietto et al. (2015) who found, in a HCW (3 VF beds + 1 HF bed) that treated artificial wastewater, seasonal variations performance with lower effluent NO$_3$–N concentrations during the warm period (higher temperature). The nitrate RE was always positive showing a reduction ranging between 18.5% (autumn 2012) and 66.9% (summer 2013) in SSL and between 88.2% (spring...
2013) and 99.3% (summer 2013) in FWTL system (Tab.5). The NO$_3$-N ARL, for all monitoring periods, was below or equal to 1.0 g m$^{-2}$ d$^{-1}$ in both SSL and FWTL (Tab.6) with an average NO$_3$-N daily load of 1.5 g m$^{-2}$ in SSL and 0.2 g m$^{-2}$ in FWTL. Comino et al. (2013), in a small HCW (2 VF beds + 1 HF bed), reported an ARL of 0.75 g m$^{-2}$ d$^{-1}$ and 2.06 g m$^{-2}$ d$^{-1}$ with an average NO$_3$-N daily load of 4.0 g m$^{-2}$ and 2.4 g m$^{-2}$ respectively.

**Phosphorus forms**

The inlet median value of TP ranged from 31.6 mg L$^{-1}$ (autumn 2012) to 43.4 mg L$^{-1}$ (spring 2013) instead PO$_4$-P ranged from 29.3 mg L$^{-1}$ (spring 2013) to 30.7 mg L$^{-1}$ (summer 2012). The SSL in 2012 and SSL + FWTL in 2013 showed a significant concentration reduction than diluted DFF inlet values for both TP and PO$_4$-P (Fig.7 and Fig.8).

![Box-plot diagrams of wastewater TP concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

**Figure 7** – Box-plot diagrams of wastewater TP concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.
Considering TP and PO$_4$-P SSL concentration abatement the higher values were reached in spring 2013, the lower ones in autumn 2012; FWTL showed an abatement lower than 15% for both phosphorus forms (Tab.4). The TP RE, considering both SSL and FWTL, ranged between 29.2% and 59.6% (Tab.5) with an ARL ranging from 0.1 g m$^{-2}$ d$^{-1}$ to 1.2 g m$^{-2}$ d$^{-1}$ (Tab.6). The two treatment lines RE of PO$_4$-P ranged from 30.5% to 60.6% (Tab.5) and the ARL from 0.1 g m$^{-2}$ d$^{-1}$ to 0.9 g m$^{-2}$ d$^{-1}$ (Tab.6).

Very few data are available in the literature about P removal using HCW. Comino et al. (2013), in a HCW that treated digestates, reported a PO$_4$-P ARL ranging from 0.2 g m$^{-2}$ d$^{-1}$ to 1.9 g m$^{-2}$ d$^{-1}$. Comino et al. (2011), in a HCW used to treat cheese factory wastewater, found an average TP concentration abatement of 57.5%. Borin et al. (2013b), in a HCW that treated pig slurry effluent, reported a TP ARL of 0.54 g m$^{-2}$ d$^{-1}$. The mechanisms by which phosphorus is removed from wastewater include sorption on substrates, storage in biomass and precipitation (Vymazal, 2007). In this study, considering the high diluted DFF pH, phosphorus removal was probably mainly due to

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**Figure 8** – Box-plot diagrams of wastewater PO$_4$-P concentration in the hybrid system sampling points and periods. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.
adsorption and/or precipitation on suspended solid, as indirectly confirmed by the positive correlation ($p<0.00001$) between the NTU presence and TP concentration in DFF (Spearman R = 0.69) and PO$_4$-P (Spearman R = 0.65).

**GHGs emission**

CWs vegetation presence and management can play an important role on GHGs emission. In our study the CH$_4$ emission from SSL beds was significantly increased (about 8 times) by plant cutting (Fig.9) which was done when shoots were not yet completely dried.

![Box-plot diagram of SSL beds CH$_4$ emission before and after plant cutting in autumn 2012.](image)

**Figure 9** – Box-plot diagram of SSL beds CH$_4$ emission before and after plant cutting in autumn 2012. Different letters indicate significant differences at $p < 0.05$ by Mann-Whitney test.

The data are in agreement with Zhu et al. (2007) and Barbera et al. (2014b) who reported the same trend in a sub-surface flow CW for *P. communis* and *Miscanthus x giganteus* respectively. The CH$_4$ emission increase was probably due to the lower O$_2$ release into the rhizosphere after cutting that reduced CH$_4$ oxidation and improved anoxic conditions, promoting CH$_4$ production. In fact O$_2$ transport through the plant into the rhizosphere was mainly controlled by plant photosynthetic activity (Caffrey and Kemp, 1991). As reported in Brix et al. (1996) for *P. australis*, green shoots are influx culms with a net O$_2$ flux to the below-ground organs and sediment up to 5.7 L m$^{-2}$ d$^{-1}$ whereas dead, broken or damaged shoots are efflux culms. Brix et al. (1992) reported that internal pressurization and convective through-flow of air are common mechanisms of
internal gas transport for many wetland species with cylindrical culms and linear leaves, finding a connective air flow rate of $5.29 \pm 0.40$ cm$^3$ m$^{-1}$ culm$^{-1}$ and < 0.01 cm$^3$ m$^{-1}$ culm$^{-1}$ for *P. australis* and *A. donax* respectively. Therefore in the bed vegetated with *A. donax* the greater effect on CH$_4$ oxidation can be due not so much to the lower oxygen released but to its disappearance after plant cutting that reduced oxygen diffusion in the substrate promoting conditions for CH$_4$ production. This last consideration is in agreement with the CH$_4$ emission data reported in Table 6. In fact the *A. donax* rhizomes did not regrow in spring 2013 determining an average CH$_4$ emission of 6,126.2 mg m$^{-2}$ h$^{-1}$ whereas a new transplanting, done in the first week of June 2013, determined a reduction of about 80% in the average CH$_4$ emission after 6 weeks. Similarly, a higher environmental impact of digestate depuration was shown in spring than summer 2013 with greater CH$_4$ plant emission/COD plant inlet (%) ratio and CH$_4$ plant emission/COD plant reduction (%) ratio (Tab.7). No significantly different CH$_4$ emissions were detected for each bed between the two studied seasons, while comparing the three beds significantly higher emissions were detected in the VF bed vegetated with *A. donax* with respect to the others in both seasons.

Seasons and SSL beds did not statistically influence CO$_2$ emissions, with an average CO$_2$ emission from the SSL of 4539.8 mg m$^{-2}$ h$^{-1}$ and 4261.5 mg m$^{-2}$ h$^{-1}$ in spring and summer 2013 respectively. The CO$_2$ beds emission/COD inlet (%) ratio was about 10% higher in summer than in spring, whereas the CO$_2$ beds emission/COD reduction (%) ratio was 5% higher in summer than in spring (Tab.7).

N$_2$O emissions were not significantly different among the SSL beds in the same season. Considering each bed in the two seasons, no significantly different N$_2$O emissions were found for VF beds whereas the HF bed showed a significantly lower emission in summer compared to spring. This last result can due to the lower wastewater Eh values in the HF bed in the summer (Tab.3). Lower wastewater Eh in the summer was also measured in the VF beds and probably due to the higher root exudate released, which generally ranged from 5% to 25% (Brix, 1997), by the plants at their maximum growth phase, which depleted oxygen availability for higher microorganisms growth (Andrews and Harris, 2000; Karjalainen et al., 2001). However, the alternative load in VF beds determined a higher oxygen presence that maintained emissions at the same levels also
in summer. Maltais-Landry et al. (2009a,b), in laboratory-scale HF CWs, reported that the beds artificial aeration, and so higher presence of oxygen, determined an increased N\(_2\)O emission. Mander et al. (2011) using fluctuating water table HF CWs that brought intermittent oxygen to the beds found an increased N\(_2\)O emission due to both nitrification and denitrification. The SSL lower N\(_2\)O-N beds emission/TN inlet (%) ratio and N\(_2\)O-N beds emission/TN reduction (%) ratio were found in summer (Tab.7) probably due to higher root exudate release in agreement with Kozub and Liehr (1999) who reported that denitrification in the wetland was limited by the availability of easily degradable sources of organic carbon.
<table>
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<tr>
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<th>CO₂ emission (mg m⁻² h⁻¹)</th>
<th>CO₂ emission (g d⁻¹)</th>
<th>COD inlet (g d⁻¹)</th>
<th>COD reduction (g d⁻¹)</th>
<th>CO₂ emission / COD inlet (%)</th>
<th>CO₂ emission / COD reduction (%)</th>
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<td>VF P. australis</td>
<td>VF A. donax</td>
<td>HF P. australis</td>
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<td>Average</td>
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<tr>
<td>Spring 2013</td>
<td>4013.2 (+1473.3)</td>
<td>5070.4 (+3664.5)</td>
<td>4535.7 (+962.8)</td>
<td>1634.3</td>
<td>4432.4</td>
<td>2566.4</td>
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<td>4599.1 (+5168.2)</td>
<td>2917.8 (+2470.1)</td>
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<td></td>
<td>CH₄ emission (mg m⁻² h⁻¹)</td>
<td>CH₄ emission (g d⁻¹)</td>
<td>COD inlet (g d⁻¹)</td>
<td>COD reduction (g d⁻¹)</td>
<td>CH₄ emission / COD inlet (%)</td>
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<td>Average</td>
<td>Average</td>
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<tr>
<td>Spring 2013</td>
<td>129.6 (+368.3)</td>
<td>6126.2 (+10607.5)</td>
<td>15.4 (+31.5)</td>
<td>752.5</td>
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<td>N₂O emission (mg m⁻² h⁻¹)</td>
<td>N₂O-N emission (g d⁻¹)</td>
<td>TN inlet (g d⁻¹)</td>
<td>TN reduction (g d⁻¹)</td>
<td>N₂O-N emission / TN inlet (%)</td>
<td>N₂O-N emission / TN reduction (%)</td>
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<td>Spring 2013</td>
<td>6.5 (+8.6)</td>
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<td>Summer 2013</td>
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<td>11.0 (+11.6)</td>
<td>2.4 (+3.2)</td>
<td>2.20</td>
<td>284.1</td>
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Conclusions

The aim of this research was to evaluate the depuration performance and GHGs emission from a pilot HCW that treated diluted DFF in north-east Italy. HCW was loaded with diluted digestate therefore in a full scale CW a pre-treatment stage should be expected. Although the HCW inlet was diluted DFF, during experimental periods, the plant was managed fixing severe load condition with high pollutant inflow concentration, especially for COD (from 4,580 to 6,000 mg L\(^{-1}\)) and TN (from 378.0 to 657.5 mg L\(^{-1}\)) that was mainly represented by NH\(_4\)-N. SSL showed better depuration performance than FWTL with an average areal load reduction of COD, TN, NH\(_4\)-N and NO\(_3\)-N of 134.8, 14.3, 9.3 and 0.6 g m\(^{-2}\) d\(^{-1}\) in the SSL beds and 13.8, 1.0, 0.6 and 0.2 g m\(^{-2}\) d\(^{-1}\) in FTW basins. TP and PO\(_4\)-P diluted DFF concentration was significantly abated in both SSL and FWTL with a percentage mass removal that reached 59.6% for TP and 60.6% for PO\(_4\)-P.

Concerning VF beds vegetation, *P. australis* showed a better growth performance than *A. donax* although the two species did not show significantly different pollutant abatement values. *A. donax* did not regrow in the second year determining an increase in plant CH\(_4\) emission. CO\(_2\) emissions did not show significant differences between seasons or subsurface flow beds. The N\(_2\)O-N emission from SSL beds was 1.27% and 0.87% of TN removal and inlet, respectively.
Chapter VII

$\text{CO}_2$ emissions from agricultural soil after splash-plate digestate fluid fraction spreading and maize biomass production
Abstract

The aim of this work was to evaluate the effects of both soil texture and preparatory tillage on soil CO₂ emission after digestate fluid fraction (DFF) spreading. The study was carried out in 2014 in two open fields, located at Terrasa Padovana (Farm 1) and Bovolenta (Farm 2), in Veneto Region, Italy. Soil CO₂ emission after digestate spreading was evaluated comparing on farm 1 the effect of soil texture (sandy loam vs clay loam) and on farm 2 the effect of soil preparatory tillage (ripping vs plowing) in clay loam soil. Un-amended soil was considered as control. DFF was supplied, using splash-plate technique, at a dose equal to 170 kg total nitrogen ha⁻¹, on March 14th and 20th respectively on farm 1 and farm 2. On April 12th, on both farms, maize (Zea mais L.) was sown at a density of 7.5 plants m⁻². About 3 days after DFF distribution soil CO₂ fluxes were not significantly different from un-amended plots except for a few hours after first soil tillage and a rainfall event. On the average of the two weeks after digestate spreading, on both farms, soil CO₂ emissions were significantly higher in the amended plots than un-amended ones with a median value of about 1.6 and 3.4 times greater, respectively on farm 1 and farm 2. In those 15 days, the amount of C supplied to the soil by DFF emitted as CO₂-C was 34.1% in the sandy loam soil whereas it ranged from 9.6% to 20.2% in the clay loam. During maize growing season, only on farm 1, soil CO₂ emission was significantly higher in the amended plots than un-amended ones without soil texture influence. In the same period DFF spreading and soil preparatory tillage exerted no significant effects on soil CO₂ emissions in farm 2, probably due to the high N mineral fertilization supplied in May that reduced microorganism activity. DFF determined no significant effect on maize yield in farm 1 whereas in farm 2 a higher yield was found in amended treatments (+17%) than un-amended ones, probably influenced by no K mineral fertilization and soil waterlogging.

Introduction

In Italy biogas production represents the most widespread energy source obtained from biomass, thanks to the specific legislative tools aimed at increasing its production in the various economic sectors involved, ranging from livestock farming to agro-industrial (Tricase and Lombardi, 2009). The biogas production plants, fed with livestock effluent
and manure and biomasses, have a potentially double advantage, reducing the environmental impact of animal waste and providing clean energy. In recent years, there has been a proliferation of biogas energy plants in Italy, reaching 1,054 in 2013, mainly located in the Po Valley (Carrosio, 2013). The great majority of biogas plants (94.3%) are managed by farmers who use energy crops biomass and/or livestock manure as raw materials (Fabbri et al., 2013). The sustainability of biogas production may depend on an appropriate end-use of the digested material obtained (de la Fuente et al., 2013), which can be treated (Florio et al., 2012; Maucieri et al., 2013) or re-used in a suitable way to reduce its negative environmental impact. During the anaerobic digestion process, the original feedstock biomass undergoes several composition changes that are relevant for plants nutrients availability after field digestate spreading (Möller and Müller, 2012). Mainly total organic carbon contents decrease and nitrogen (N) concentration increases (Tambone et al., 2009).

Several authors reported positive fertilizer effects from digestate application on crops (Montemurro et al., 2010; Haraldsen et al., 2011; Alburquerque et al., 2012a), replacing inorganic fertilizer use with less impact on the environment (Walsh et al., 2012). The application of organic materials to agricultural soil is a good practice to improve or maintain agro-ecosystems environmental sustainability (Haynes and Naidu, 1998; Morari et al., 2006; Fecondo et al., 2008; Diacono and Montemurro, 2010; Barbera et al., 2013; Migliorini et al., 2014). At the same time it is also well known that organic matter (OM) application on soil can increase greenhouse gas emission, such as carbon dioxide (CO₂) (Li et al., 2013). However the application of appropriate agronomic management techniques regarding organic soil amendment represents one of the best opportunities for greenhouse gas mitigation (Pezzola et al., 2012) maintaining the positive effect of OM on soil fertility. Several laboratory studies investigated the effect of digestate application on soil CO₂ emission (Cayuela et al., 2010; Grigatti et al., 2011; Marchetti et al., 2012; Sänger et al., 2013; de la Fuente et al., 2013; Johansen et al., 2013) but only a few monitored soil respiration in open field conditions (Pezzola et al., 2012).
The aim of this work was to evaluate in the open field the effect of both soil texture and preparatory tillage on soil CO$_2$ emission after digestate fluid fraction (DFF) splash-plate spreading.

**Materials and Methods**

**Site description and experimental design**

The open-field study was performed in 2014 on two farms located in Terrasa Padovana (Farm 1) and Bovolenta (Farm 2), in Veneto Region, Italy. Farm 1 had two different soil textures, sandy loam and clay loam, whereas farm 2 had only clay loam soil (USDA 1999). The main soil chemical characteristics, determined before starting the experiment, are reported in table 1.

<table>
<thead>
<tr>
<th>Table 1 – Soil chemical characteristics (mean value ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm 1</td>
</tr>
<tr>
<td>Sandy loam</td>
</tr>
<tr>
<td>pH</td>
</tr>
<tr>
<td>EC (µS cm$^{-1}$)</td>
</tr>
<tr>
<td>OC (g kg$^{-1}$)</td>
</tr>
<tr>
<td>NTK (mg kg$^{-1}$)</td>
</tr>
<tr>
<td>NO$_3$-N (mg kg$^{-1}$)</td>
</tr>
<tr>
<td>NO$_2$-N (mg kg$^{-1}$)</td>
</tr>
<tr>
<td>NH$_4$-N (mg kg$^{-1}$)</td>
</tr>
<tr>
<td>PO$_4$-P (mg kg$^{-1}$)</td>
</tr>
<tr>
<td>P (g kg$^{-1}$)</td>
</tr>
<tr>
<td>Ca (g kg$^{-1}$)</td>
</tr>
<tr>
<td>K (g kg$^{-1}$)</td>
</tr>
<tr>
<td>Mg (g kg$^{-1}$)</td>
</tr>
<tr>
<td>Na (g kg$^{-1}$)</td>
</tr>
</tbody>
</table>

On farm 1 the effect of soil texture on soil CO$_2$ emission after DFF spreading was studied through the follow four treatments: sandy loam soil with digestate (SL-D), sandy loam soil without digestate (SL-ND), clay loam soil with digestate (CL-D) and clay loam soil without digestate (CL-ND). On farm 2 the effect of preparatory tillage on soil CO$_2$ emission after DFF spreading was evaluated through four treatments: ripping soil with digestate (RS-D) and without digestate (RS-ND), plowing soil with digestate (PS-D) and without digestate (PS-ND). On each farm a randomized block design replicated twice
with experimental plot of about 800 m² was used. DFF, obtained from anaerobic
digestion of cattle slurry and manure, maize silage and flour, was spread by splash-plate
technique on March 14th (Farm 1) and on March 20th (Farm 2) in a volume to supply 170
kg total nitrogen ha⁻¹. The main digestate physical-chemical characteristics, determined
in three samples before the spreading operation, are reported in table 2.

Table 2 – Digestate physical-chemical characteristics (mean value ± SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC (mS cm⁻¹)</td>
<td>25.23 ± 0.50</td>
</tr>
<tr>
<td>Redox potential (mV)</td>
<td>-437.50 ± 35.75</td>
</tr>
<tr>
<td>pH</td>
<td>7.40 ± 0.05</td>
</tr>
<tr>
<td>COD (g L⁻¹)</td>
<td>44.07 ± 0.67</td>
</tr>
<tr>
<td>BOD₅ (g L⁻¹)</td>
<td>3.77 ± 0.21</td>
</tr>
<tr>
<td>Specific weight (g L⁻¹)</td>
<td>1000.56 ± 0.41</td>
</tr>
<tr>
<td>Dry matter %</td>
<td>4.82 ± 0.27</td>
</tr>
<tr>
<td>C (% DM)</td>
<td>36.34 ± 2.50</td>
</tr>
<tr>
<td>S (% DM)</td>
<td>1.00 ± 0.36</td>
</tr>
<tr>
<td>Total N (% DM)</td>
<td>3.89 ± 0.40</td>
</tr>
<tr>
<td>TKN (% FM)</td>
<td>0.49 ± 0.002</td>
</tr>
<tr>
<td>NH₄-N (% FM)</td>
<td>0.34 ± 0.005</td>
</tr>
<tr>
<td>NO₃-N (mg kg⁻¹ FM)</td>
<td>17.94 ± 1.62</td>
</tr>
<tr>
<td>NO₂-N (mg kg⁻¹ FM)</td>
<td>1.47 ± 0.33</td>
</tr>
<tr>
<td>PO₄-P (mg kg⁻¹ FM)</td>
<td>422.37 ± 7.47</td>
</tr>
<tr>
<td>P (mg kg⁻¹ FM)</td>
<td>537.30 ± 27.26</td>
</tr>
<tr>
<td>K (mg kg⁻¹ FM)</td>
<td>3072.21 ± 35.81</td>
</tr>
<tr>
<td>Ca (mg kg⁻¹ FM)</td>
<td>895.56 ± 17.24</td>
</tr>
<tr>
<td>Na (mg kg⁻¹ FM)</td>
<td>222.42 ± 2.54</td>
</tr>
<tr>
<td>Mg (mg kg⁻¹ FM)</td>
<td>590.03 ± 25.81</td>
</tr>
</tbody>
</table>

After spreading, in both farms, two superficial harrowings were done to prepare seedbeds,
and on April 12th maize (Zea mais L.) was sown at a density of 7.5 plants m⁻². Between
the end of April and end of May two mechanical weedings were done. On farm 2, maize
was flooded from April 28th to May 2nd, due to heavy rain (about 141 mm) and clay
loam soil texture.

The mineral fertilizer supply differed between the two farms. In the first one, with the
second harrowing, before sowing, 40 kg N ha⁻¹ and 100 kg ha⁻¹ of P and K were
distributed; a further 50 kg N ha⁻¹ was supplied with the first weeding after sowing. On
farm 2, 45 kg N ha⁻¹ and 115 kg P ha⁻¹ were added to soil before sowing with the second
harrowing. In farm 2, at each of the two weedings after sowing, 115 kg N ha\(^{-1}\) was added.

**Soil CO\(_2\) flux measurement**

CO\(_2\) fluxes were measured with the static non-stationary chamber technique (Barbera et al., 2014a; Maucieri et al., 2014b) using a chamber with a volume of 5 L and 10 cm square base. CO\(_2\) emissions were detected in three points of each experimental plot in order to replicate the measures in the space. After digestate spreading (1\(^{st}\) monitoring period) soil CO\(_2\) emission was measured 9 times on farm 1 (from 1 to 339 hours) and 6 times on farm 2 (from 1 to 270 hours). After maize emergence (2\(^{nd}\) monitoring period) it was measured 7 times on farm 1 (from 30 to 112 days) and 6 times on farm 2 (from 30 to 100 days). Soil CO\(_2\) flux was determined by measuring the temporal change in CO\(_2\) concentration inside the chamber using a portable IR instrument (Geotech G150) detecting CO\(_2\) concentrations at levels of parts per million.

CO\(_2\) flux was calculated using the following formula:

\[
\text{CO}_2 = \frac{V}{A} \cdot \frac{dc}{dt}
\]

where CO\(_2\) flux is expressed in mg CO\(_2\) m\(^{-2}\) s\(^{-1}\); V (m\(^3\)) is the volume and A (m\(^2\)) the footprint of the flux chamber; ‘c’ is the CO\(_2\) concentration (mg CO\(_2\) m\(^3\)) and ‘t’ the time step (s).

In each CO\(_2\) measurement point temperature and moisture in the first 7.5 cm soil layer (TDR 100 Soil Moisture Meter) were also detected.

Some authors reported that, in maize-grown soil, CO\(_2\) fluxes measured between 9:00 and 12:00 am represent the mean CO\(_2\) daily emissions (Rochette and Flanagan, 1997; Lou et al., 2004; Ding et al., 2006) in view of this in our study soil CO\(_2\) emission measures, during maize growing season, were carried out between these hours. To compare cumulative soil CO\(_2\)-C emission with the amount of C supplied to the soil by DFF during the first monitoring period, the cumulative CO\(_2\)-C emission value monitored in the ND treatment was subtracted from the same amended treatment.

**Maize biomass measurement**

The maize aboveground biomass was harvested on August 6\(^{th}\) and 7\(^{th}\) respectively on farm 2 and farm 1 at waxy ripeness. In each experimental plot, fresh biomass production was
measured in four points (each 1.5 m x 4 m) for a total of 8 replicated production areas per treatment. Areas were selected randomly and maize plants were cut manually at 10 cm from soil. Biomass dry weight was determined by drying plant samples in a thermo-ventilated oven at 65 °C until constant weight was reached.

**Statistical analysis**

The normality of CO$_2$ data was checked using the Kolmogorov–Smirnov test, because they didn’t show normal distribution the Kruskal–Wallis and Mann-Whitney non-parametric tests were used to check the significance of differences (accepted at the level of p<0.05). Correlations between soil temperature and moisture with CO$_2$ emissions were evaluated using Spearman Rank correlation.

Statistical analysis of biomass production was conducted by ANOVA and mean values were compared using Fisher LSD test.

**Results and Discussion**

**Soil CO$_2$ emissions**

The effect of DFF on soil CO$_2$ emission trend, during the first monitored period, was described by the Harris model on both farms (Tab.3). It was characterized by high emissions immediately after spreading (1 hour), due to both the release of CO$_2$ dissolved in the digestate and the rapid microorganism respiration of easily degradable carbon (Bol et al., 2003; Faguerio et al., 2010), and a fast CO$_2$ flux decrease during the first 3 days after spreading.

**Table 3** – Harris equation model and regression coefficient ($r^2$) of the soil digestate CO$_2$ emission fitted function in the three experimental condition.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Soil texture</th>
<th>Equation</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sandy loam</td>
<td>CO$_2$ = 1 / (a + bx$^2$)</td>
<td>9.44E-02</td>
<td>4.70E-03</td>
<td>1.41E+00</td>
<td>0.998</td>
</tr>
<tr>
<td>1</td>
<td>Clay loam</td>
<td>CO$_2$ = 1 / (a + bx$^2$)</td>
<td>2.84E-01</td>
<td>3.68E-04</td>
<td>2.11E+00</td>
<td>0.996</td>
</tr>
<tr>
<td>2</td>
<td>Clay loam</td>
<td>CO$_2$ = 1 / (a + bx$^2$)</td>
<td>1.45E-01</td>
<td>3.08E-02</td>
<td>1.32E+00</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Rapid CO$_2$ emission decrease after soil digestate application was also obtained in laboratory experiments by Sänger et al. (2011), Grigatti et al. (2011) and de la Fuente et al. (2013). On farm 1 DFF spreading, in both soil textures, determined a significantly
higher CO₂ emission increase than un-amended plots in the first 71 hours (Fig.1 and Fig.2).

**Figure 1** – Box-plot diagram of Farm 1 CO₂ emissions during the 339 hours after spreading on sandy loam soil. Different letters indicate significant differences at p < 0.05 by Mann-Whitney test between amended and un-amended plots in the same monitored hour.
Figure 2 – Box-plot diagram of Farm 1 CO₂ emissions during the 339 hours after spreading on clay loam soil. Different letters indicate significant differences at p < 0.05 by Mann-Whitney test between amended and un-amended plots in the same monitored hour.

Furthermore, significant CO₂ fluxes increases in the amended plots than un-amended ones, although always with median value lower than 0.5 g m⁻² h⁻¹, were determined both by: 1) first harrowing (+1.8 times in clay loam soil and +2.5 times in sandy loam; 120 hours after spreading) that made buried organic material available to microorganisms; 2) rainfall event (+1.7 times in clay loam soil and +2.8 times in sandy loam; 19.6 mm from about 190 to 240 hours after spreading) that improved the soil condition for microorganism activities. On farm 2, during the first monitoring period soil preparatory tillage did not have any effect on soil CO₂ emission (Fig.3) showing, on the average of soil tillage, a soil CO₂ emission trend (Fig.4) similar to those monitored on farm 1. Up to 150 hours after spreading, CO₂ flux was significantly higher in the amended plots than un-amended ones probably due to a rainfall event (19.6 mm from about 50 to 100 hours after spreading). The first soil harrowing, that was done after the rainfall event (10 days
after spreading), determined a significantly higher soil CO$_2$ flux (3.4 times) from amended plots than un-amended ones.

**Figure 3** – Box-plot diagram of Farm 2 soil CO$_2$ emissions in the first monitored period. RS-D = ripping soil with digestate; RS-ND = ripping soil without digestate; PS-D = plowing soil with digestate; PS-ND = plowing soil without digestate. Different letters indicate significant differences at p < 0.05 by Kruskal-Wallis test.

**Figure 4** – Box-plot diagram of Farm 2 soil CO$_2$ emissions during the 270 hours after spreading on the average of soil tillage. Different letters indicate significant differences at p < 0.05 by Mann-Whitney test between amended and un-amended plots in the same monitored hour.
Significantly higher CO₂ emission on farm 1, considering the 1st monitored period values, was detected in sandy loam soil than clay loam one, with or without DFF spreading (Fig.5), in agreement with Hébert et al. (1991) who evaluated different composts C mineralization in sandy or loamy soils finding a higher C mineralization in the former. As known sandy loam soils have higher relative organic C loss than clay soil (Burke et al., 1989), in fact this latter soil type accumulates more organic matter than sandy soil because organic matter is stabilized to a greater degree and less accessible to microbial decomposition. Van Veen et al. (1989) reported that soil structure and texture had a large effect on the easily degradable organic carbon turnover through the microbial biomass, with clayey soils tending to be more 'preservative' than coarser, sandy soils.

Figure 5 – Box-plot diagram of Farm 1 soil CO₂ emissions in the first monitored period. SL-D = Sandy loam soil with digestate; SL-ND = Sandy loam soil without digestate; CL-D = clay loam soil with digestate; CL-ND = clay loam soil without digestate. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

Considering all CO₂ fluxes data recorded during the first monitored period, on the average of soil texture (Farm 1) and tillage (Farm 2), in both farms, soil CO₂ emission was significantly higher by about 1.6 (Farm 1) and 3.5 (Farm 2) times in the amended plots than un-amended ones, which showed a median value of 0.25 g m⁻² h⁻¹ (Farm 1) and 0.04 g m⁻² h⁻¹ (Farm 2). Monitored data are in agreement with Pezzola et al. (2012) who reported that the application of digestate led to an increase in soil CO₂ emission.
The C supplied to the soil with digestate was about 158.8 g m$^{-2}$. Cumulative soil CO$_2$-C emission of C supplied to the soil by DFF, calculated by the Harrison model equation, during 339 and 270 hours after spreading respectively on farm 1 and farm 2, was 34.1% of C supplied in the sandy loam soil (Farm 1), and ranged from 9.6% (Farm 2) and 20.2% (Farm 1) in the clay loam one. Cayuela et al. (2010), in a laboratory experiment, adding to a sandy soil digestate at rate of 150 kg N ha$^{-1}$ after 60 days incubation at 20 °C, reported that 40% of applied C was emitted as CO$_2$.

During maize growing season, on farm 1, soil CO$_2$ emission was significantly higher (1.7 times) in the amended plots than un-amended ones, which showed an emission median value of 0.29 g m$^{-2}$ h$^{-1}$; no influence was detected for soil texture (Fig.6). Instead on farm 2, no differences were detected among experimental plots with a median CO$_2$ emission value lower than 0.4 g m$^{-2}$ h$^{-1}$ (Fig.7).

![Figure 6](image.png)

**Figure 6** – Box-plot diagram of Farm 1 soil CO$_2$ emissions during maize growing season. SL-D = Sandy loam soil with digestate; SL-ND = Sandy loam soil without digestate; CL-D = clay loam soil with digestate; CL-ND = clay loam soil without digestate. Different letters indicate significant differences at $p < 0.05$ by Kruskal–Wallis test.
The absence of significant differences in CO₂ emission among amended and un-amended treatments on farm 2 was probably due to the high N mineral fertilization added in May that had caused soil secondary salinization and acidification and reduced microorganism biomass (Lee and Jose, 2003) and activity (Shen et al., 2013), although a residual organic fraction supplied with digestate was still available in amended plots. Ding et al. (2010) reported conflicting evidence in the literature about the effect of N fertilization on soil CO₂ emission with either increase (Liljeroth et al., 1990; Gallardo and Schlesinger, 1994) or decrease (Cardon et al., 2001; Giardina et al., 2004). Comparing the two farms clay loam soils, the significantly higher CO₂ emission was shown by amended plots on farm 1 (Fig.8). No significant differences were found among un-amended plots on farm 1 and both amended and un-amended plots on farm 2; therefore high N mineral fertilization reduced soil CO₂ emission rate of farm 2 amended plots whereas no effect seemed to be exerted on un-amended plots CO₂ emission comparing the two farms.
During the first monitored period, on farm 1, soil CO$_2$ emission was positively correlated with soil moisture (p<0.05) only in sandy loam soil in both amended (Spearman R=0.299) and un-amended (Spearman R = 0.290) plots. The effect of soil moisture is in agreement with Dilustro et al. (2005) who found that soil CO$_2$ emissions were significantly related with soil moisture in sandy soils but not in clayey soils when soil water content was above the wilting point threshold. In our case, average soil moisture during the monitoring period in clay loam soil was 22.3 ± 2.3%. On this farm in same monitoring period soil CO$_2$ emission was positively correlated with soil temperature (p<0.001) only in un-amended clay loam texture soil (Spearman R = 0.436). Soil CO$_2$ emission from amended clay loam texture soil was not correlated with soil temperature because of an emission peak in the first 23 hours after spreading. In fact without this dataset values also in amended treatment soil CO$_2$ emission was positively correlated with soil temperature (p<0.05; Spearman R = 0.320). No correlations were found during maize growing season on farm 1 and during both monitoring periods on farm 2.
Maize biomass production

DFF spreading did not significantly influence the maize aboveground dry biomass on farm 1, with an average production of 22.7 Mg ha\(^{-1}\) and 18.7 Mg ha\(^{-1}\) in clay loam and sandy loam soils, respectively. Instead, on farm 2, a significant (p<0.05) effect was found with a higher yield in the amended plots (+17%) than un-amended ones that produced 18.0 Mg ha\(^{-1}\). The different response to digestate application, comparing the two farms, was probably due to: 1) K mineral fertilization that was applied only on farm 1. In fact, on farm 2 the K content was only supplied in digestate at a dose equal to 106.5 kg ha\(^{-1}\). Niu et al. (2011) found that summer maize grain yields increased from 9.9% to 14.9% in K fertilizer treatments compared with K unfertilized ones. He et al. (2012), in a long-term study, reported that K fertilizer application alone significantly improved maize yield by 46%. Qiu et al. (2014) in a 20 years study found that maize K fertilization significantly increased the average grain yields by 15.1% and 13.8% supplying 93.8 and 186.8 kg K ha\(^{-1}\) year\(^{-1}\) respectively; 2) the 5 days of flooding that occurred on farm 2 highlighted even more the difference between amended and un-amended plots. In fact, they occurred when maize was more susceptible (Zaidi et al., 2004) and probably determined a deficiency of essential macronutrients (N, P and K) and an accumulation of toxic nutrients resulting from decreased plant root uptake and change in redox potential (Cairns et al., 2012).

Conclusions

After DFF spreading, soil CO\(_2\) emission was significantly higher in the amended plots than un-amended ones for about 3 days on both farms. Digestate effect on soil CO\(_2\) flux was described by the Harris model with high emissions immediately after spreading followed by a fast decrease in the first 3 days. Soil preparatory tillage did not show any effect on soil CO\(_2\) emission, whereas sandy loam soil showed significantly higher CO\(_2\) emission than clay loam in the two weeks after spreading. During maize growing season soil CO\(_2\) emission was significantly higher in the amended plots than un-amended ones only on farm 1 irrespective of soil texture influence. No significant effect on soil CO\(_2\) emission was found for amendment and soil preparatory tillage on farm 2 probably due to the high N fertilization applied in May that reduced microorganism activity.
No significant maize yield difference was determined by DFF on farm 1 whereas on farm 2 a higher yield was found in amended treatments (+17%) than un-amended ones, probably influenced by no K mineral fertilization and soil waterlogging. Although the results reported in this thesis concern data from only one year, and further experiments are needed in order to reduce soil CO$_2$ from digestate amended soil, they indicate clay loam soil as more suitable for digestate spreading independently of preparatory tillage, plowing or ripping.
Chapter VIII
Effect of digestate fluid fraction injection depth on soil carbon dioxide emission and maize biomass production
Abstract

The aim of this study was to evaluate, in open field conditions, the effect of digestate fluid fraction injection depth (10 cm, 25 cm and 35 cm) in clay loam soil, on CO₂ emission. An un-amended soil was considered as control. The study was performed in 2014 on a farm located in Terrasa Padovana, Veneto Region (Italy) distributing digestate before maize sowing.

Soil digestate injection determined a high CO₂ emission in the first hour after application followed by a progressive reduction as early as 24 hours that reached significantly lower values, similar to those measured in the un-amended control, after 48 hours. Comparing the gas soil emissions (measured 1 hour after digestate application) with soil injection depth an opposite trend is observed, in which CO₂ flux decreases as soil injection depth increases with significantly higher emission values in the 10 cm treatment (median value 23.7 g CO₂ m⁻² h⁻¹) than the 35 cm one (median value 2.5 g CO₂ m⁻² h⁻¹). In the 3 days between digestate distribution and maize sowing soil CO₂ emission was significantly higher in the amended treatments than un-amended one, with median values of 1.53 g CO₂ m⁻² h⁻¹ and 0.46 g CO₂ m⁻² h⁻¹ respectively. During maize growing season no significant soil CO₂ emission differences were monitored among treatments with a median value of 0.33 g CO₂ m⁻² h⁻¹.

Digestate application significantly improves maize aboveground dry biomass with an average yield of 22.0 Mg ha⁻¹ and 16.2 Mg ha⁻¹ in amended and un-amended plots respectively.

Introduction

Intensive soil fertilization with mineral fertilizers has led to several issues, like loss of soil carbon (C) and nitrogen (N) leaching (Borin et al., 1997; Nardi et al., 2004, Morari et al., 2006). Organic fertilization with organic wastes therefore represents an alternative for sustainable agriculture (Casacchia et al., 2012; Marchetti et al., 2012; Morra et al., 2013; Barbera et al., 2013; Nkoa, 2014). In this context the agricultural reuse of digestates, organic waste products of biogas plants, should be considered. Furthermore the sustainability of biogas production may depend on an appropriate end-use of the digested material obtained, which should be treated, disposed of, or re-used in a proper
way, avoiding any possible negative environmental impact (de la Fuente et al., 2013). Digested waste materials present some advantages for their use as soil amendments in comparison with untreated wastes, such as greater microbial stability and hygiene and a higher N amount as ammonium (Holm-Nielsen et al., 2009; Alburquerque et al., 2012b; Möller and Müller, 2014). Therefore digestate can be considered as organic amendment or organic fertilizer when properly handled and managed (Nkoa, 2014). In fact the application of organic matter to agricultural soils stimulates microbial activity increasing greenhouse gases emission, hence the careful application of appropriate agriculture management techniques represents one of the best opportunities for greenhouse gases emission mitigation (Pezzolla et al., 2012).

Several laboratory scale studies investigated the effect of soil amendment with digestate on CO₂, CH₄ and N₂O emissions (Cayuela et al., 2010; Grigatti et al., 2011; Sänger et al., 2011; Alburquerque et al., 2012c; de la Fuente et al., 2013; Johansen et al., 2013). Limited number of studies reported results obtained in open field conditions, mainly focusing on N₂O and NH₃ emissions, comparing the effect of fermented and unfermented slurries or different digestate soil distribution techniques (Rubæk et al., 1996; Petersen, 1999; Wulf et al., 2002), or CH₄ emission (Dieterich et al., 2012). Only a few open field studies investigated soil CO₂ emission after digestate application, spreading it on grassland or soil surface (Pezzolla et al., 2012). To our knowledge no field experiment has been conducted to evaluate soil CO₂ emission after digestate injection at different soil depths.

With this in mind the aim of this work was to evaluate in the open field the effect of digestate fluid fraction (DFF) injection depth on clay loam soil CO₂ emission.

**Materials and Methods**

**Site description and experimental design**

The study was performed in 2014 on a farm located in Terrasa Padovana, Veneto Region, Italy on clay loam soil (USDA classification) after *Triticum aestivum* harvested at waxy ripeness. The effect of DFF injection depth on soil CO₂ emission was studied through four treatments: no digestate distribution (ND), digestate injection at 10 cm depth (10 cm), 25 cm depth (25 cm) and 35 cm depth (35 cm). Randomized block design with
three replicates and experimental plot of 500 m² was used. DFF, obtained from anaerobic digestion of cattle slurry and manure, maize silage and flour, was distributed in the soil by injection technique on June 3rd in a volume to obtain a total nitrogen soil supply of 170 kg ha⁻¹. The main chemical DFF characteristics, determined in three samples before the spreading operation, are reported in table 1. Distribution was carried out in undisturbed soil after wheat cultivation (June 3rd); after 23 hours and 45 hours respectively a weeding (25 cm depth) and harrowing (power harrow, 20 cm depth) were carried out to prepare the seedbed, and on June 6th maize (Zea mais L.) was sown at a density of 7.5 plants m².

Table 1 – Digestate chemical characteristics (mean value ± SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter %</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>TKN (mg kg⁻¹ FM)</td>
<td>2936.7 ± 8.7</td>
</tr>
<tr>
<td>NH₄-N (mg kg⁻¹ FM)</td>
<td>28.0 ± 0.9</td>
</tr>
<tr>
<td>NO₃-N (mg kg⁻¹ FM)</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>P (mg kg⁻¹ FM)</td>
<td>246.1 ± 9.2</td>
</tr>
<tr>
<td>K (mg kg⁻¹ FM)</td>
<td>2438.9 ± 14.3</td>
</tr>
<tr>
<td>Ca (mg kg⁻¹ FM)</td>
<td>621.04 ± 8.5</td>
</tr>
<tr>
<td>Na (mg kg⁻¹ FM)</td>
<td>268.7 ± 2.2</td>
</tr>
<tr>
<td>Mg (mg kg⁻¹ FM)</td>
<td>235.0 ± 7.5</td>
</tr>
</tbody>
</table>

The only mineral fertilization supplied in all experimental plots was 50 kg N ha⁻¹ as urea distributed at the same time as mechanical weed control, at the maize fifth leaf phenological stage.

**Soil CO₂ flux measurement**

CO₂ flux was measured with the static non-stationary chamber technique (Barbera et al., 2014a; Maucieri et al., 2014b) using a chamber with a volume of 5 L and 10 cm square base.

CO₂ emissions were detected in three points of each experimental plot in order to replicate the measures in the space with 9 measures for each studied treatment. After DFF distribution soil CO₂ emission was measured 3 times before maize sowing (after 1 hour in undisturbed soil and after 24 and 48 hours in the one disturbed by tillage) and 9 times after this (from 1 to 102 days). Soil CO₂ flux was determined by measuring the temporal
change in CO₂ concentration inside the chamber using a portable IR instrument (Geotech G150) detecting CO₂ concentrations at levels of parts per million.

CO₂ flux was calculated using the following formula:

\[ \text{CO}_2 = \frac{V}{A} \cdot \frac{dc}{dt} \]

where CO₂ flux is expressed in mg CO₂ m⁻² s⁻¹; \( V \) (m³) is the volume and \( A \) (m²) the footprint of the flux chamber; ‘c’ is the CO₂ concentration (mg CO₂ m⁻³) and ‘t’ the time step (s).

In each CO₂ measurement point soil layer temperature and moisture (TDR 100 Soil Moisture Meter) in the first 7.5 cm were also detected.

Some authors reported that, in maize-grown soil, CO₂ fluxes measured between 9:00 and 12:00 am represent the mean CO₂ daily emissions (Rochette and Flanagan, 1997; Lou et al., 2004; Ding et al., 2006); in view of this in our study soil CO₂ emission measures, during maize growing season, were done between these hours. Based on soil CO₂-C fluxes, the mean cumulative soil CO₂-C emission for each treatment, during both distribution phase and maize growing season, were calculated by summing the products of the average of two neighboring measurement fluxes for their interval time. To compare cumulative soil CO₂-C emission with the amount of C supplied to the soil by DFF, the cumulative CO₂-C emission value monitored in the ND treatment was subtracted from those calculated for each amended treatment.

**Maize biomass measurement**

The maize aboveground biomass was harvested on September 26th at waxy ripeness. In each experimental plot fresh biomass production was measured in four points (each 1.5 m x 4 m) for a total 12 replicated production areas for treatment. Areas were randomly selected and maize plants were manually cut at 10 cm from soil. Biomass dry weight was determined by drying plant tissue samples in a thermo-ventilated oven at 65 °C until constant weight was reached.

**Statistical analysis**

The normality of CO₂ data was checked using the Kolmogorov–Smirnov test, because they did not show normal distribution the Kruskal–Wallis and Mann-Whitney non-parametric tests were used to check the significance of differences. Correlation between soil
temperature and moisture with CO$_2$ emissions were evaluated using Spearman Rank correlation.

Statistical analysis of biomass production and cumulative CO$_2$-C emission was conducted by one-way ANOVA and mean values were compared using Fisher LSD test.

**Results and Discussion**

**Soil CO$_2$ emissions**

The DFF effect on soil CO$_2$ emission followed the same trend in all three injection depths, with a high CO$_2$ emission in the first hour after application followed by a significant reduction as early as 24 hours and that reached values similar to those measured in the un-amended control after 48 hours (Fig.1).

**Figure 1** – Box-plot diagrams of CO$_2$ emissions in the 1, 24 and 48 hours after distribution in the experimental treatments. a = digestate injection at 10 cm depth; b = digestate injection at 25 cm depth; c = digestate injection at 35 cm depth; d = un-amended control. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.
Considering the CO₂ emission trend in the 48 hours after DFF soil injection, our data are in line with studies carried out in laboratory conditions by Sänger et al. (2011) who monitored a rapid soil CO₂ production increase after biogas slurry application and by Grigatti et al. (2011) who reported, after digestate application, a very intensive CO₂ emission in the first 24 h of soil incubation followed by a reduction to a value close to the control. De la Fuente et al. (2013), again in a laboratory study, monitored a soil CO₂–C production rates rapid decrease in the days after liquid digestate application and, after three weeks CO₂ emission values similar to those measured in the control soil. Fast CO₂ soil flux in the first hour after distribution was due to both the release of CO₂ dissolved in the digestate and the rapid microorganism respiration of easily degradable C (Bol et al., 2003; Faguerio et al., 2010). In fact, as reported Johansen et al. (2013), digested residues from biogas production induced only small and transient changes on the total soil microbial biomass, function and community structure. Comparing the emissions measured 1 hour after DFF injection, CO₂ flux decreased when injection depth increase, with significantly higher emission value in the 10 cm treatment (median value 23.7 g CO₂ m⁻² h⁻¹) and the lower one in the 35 cm treatment (median value 2.5 g CO₂ m⁻² h⁻¹) (Fig.2).

![Box-plot diagram of CO₂ emissions 1 hour after digestate distribution in the experimental treatments. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

**Figure 2** – Box-plot diagram of CO₂ emissions 1 hour after digestate distribution in the experimental treatments. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.
On the average of the 3 days between DFF distribution and maize sowing, soil CO\textsubscript{2} emission was significantly higher (Mann-Whitney test, p<0.0006) in the amended treatments than un-amended one with median values of 1.53 g CO\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1} and 0.46 g CO\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1} respectively. Data are in agreement with Pezzolla et al. (2012) and Johansen et al. (2013) who reported, in an open field and laboratory experiment respectively, that after digestate application soil CO\textsubscript{2} emission increased. Among treatments the significantly higher CO\textsubscript{2} emission (Kruskal-Wallis, p<0.05) was detected in the plots where DFF was injected into the soil to a lesser depth (10 cm and 25 cm); no significantly different soil CO\textsubscript{2} emissions were found between 35 cm and ND treatments (Fig.3).

Figure 3 – Box-plot diagram of CO\textsubscript{2} emissions between digestate distribution and maize sowing in experimental plots. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.

The weeding, done 20 hours after DFF application in all four treatments, did not exert a significant effect on soil CO\textsubscript{2} emission. Instead, the harrowing done 45 hours after DFF application determined a significantly higher CO\textsubscript{2} emission (Kruskal-Wallis test, p<0.05) in the amended treatments than un-amended one, although absolute median values were lower than those measured in the previous two days. The significant effect of harrowing on CO\textsubscript{2} emission can be traced both to: 1) the higher oxygen availability in the first soil layer because of the increase in soil macroporosity, which stimulates
aerobic microbial populations; 2) the higher digestate physical accessibility for microorganisms and extracellular enzymes activities (Paustian et al., 2000).

During maize growing season no significant differences in soil CO$_2$ emission was monitored among treatments (Fig.4) with a median value of 0.33 g CO$_2$ m$^{-2}$ h$^{-1}$.

![Box-plot diagram of CO$_2$ emissions during maize growing season in experimental plots. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Kruskal–Wallis test.](image)

The C supplied to the soil by DFF was about 156.4 g m$^{-2}$. A significantly higher cumulative soil CO$_2$-C emission during the experimental period was found for 10 cm and 25 cm treatments, with an average value of 411.8 ± 63.6 g CO$_2$-C m$^{-2}$; no significant difference was found between 35 cm and ND treatments, with an average value of 301.3 ± 49.0 g CO$_2$-C m$^{-2}$ (Fig. 5).
Figure 5 – Soil cumulative CO$_2$–C emissions during experimental period. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Fisher LSD test.

Comparing cumulative soil CO$_2$–C emission with the amount of C supplied to the soil by DFF until maize sowing, the highest percentage value was detected in the 10 cm treatment with an emission of 61.4% of C supplied, followed by the 25 cm (43.8%) and 35 cm (2.2%) ones. From maize sowing to its harvest the highest soil CO$_2$–C cumulative emission was measured in the 25 cm treatment (43.6%) followed by the 35 cm (36.1%) and 10 cm (25.4%) ones. Data obtained suggest that: 1) in the short period (from digestate distribution to maize sowing) the CO$_2$–C emission decreases enhancing DFF injection depth; 2) in the long period (from digestate distribution to maize harvest), as in the short period, the lowest CO$_2$–C emission was shown by the greatest injection depth (38.3%), whereas similar values were found for 10 cm (86.8%) and 25 cm (87.4%) which therefore showed the same cumulative CO$_2$–C emission but with different proportions before and after sowing as previously reported. Considering 10 cm and 25 cm treatments, the data suggest that the injection at 10 cm is preferable to indirectly reduce CO$_2$–C release in the atmosphere because lower tractor power is required for digestate distribution. The emission values showed by DFF injection at 35 cm depth are indubitably interesting, however to reduce CO$_2$ losses in the atmosphere further studies are needed to compare soil CO$_2$ emission with the tractors CO$_2$ emission to injected digestate at different depth. During maize growing season in the first 7.5 cm soil layer, moisture ranged from 11.5% to 53.2% and temperature from 19.3 °C to 33.9 °C. Soil CO$_2$ emission was positively
correlated with both soil moisture and temperature (Tab.2), confirming the strong direct and indirect effect on organic material decomposition (Sänger et al., 2011) by soil aerobic metabolism. In our study the higher soil CO₂ emissions were monitored when soil temperature ranged from 32 to 34 °C and, at the same time, soil moisture from 21% to 26%. Results are in agreement with Suseela et al. (2012) who found that soil respiration proceeded fastest at the warmest temperatures when soil water content ranged from 20% to 30%.

Maize biomass production

DFF distribution significantly (ANOVA, p<0.05) improved maize aboveground dry biomass (Fig.6) with an average production, in amended and un-amended plots, of 22.0 Mg ha⁻¹ and 16.2 Mg ha⁻¹ respectively, confirming the high fertilizer value of DFF (Nkoa, 2014). Maize yield obtained in amended plots is in agreement with our previous data detected with DFF splash-plate spreading on a clay loam soil; we found a maize dry biomass production of 22.7 Mg ha⁻¹ without significant differences between DFF and mineral fertilization. This result confirmed that anaerobic digestates could be regarded as effective organic fertilizers (Nkoa, 2014). Furthermore, Walsh et al. (2012) reported that replacing inorganic fertilizers with liquid digestate could maintain or improve yields from grassland systems, with less impact on the environment. In our study no significant influence was exerted by digestate injection depth on maize dry biomass yield (Fig.6).

![Figure 6](image)

**Figure 6** – Maize dry biomass production at waxy ripeness. 10 cm = digestate injection at 10 cm depth; 25 cm = digestate injection at 25 cm depth; 35 cm = digestate injection at 35 cm depth; ND = plots without digestate injection. Different letters indicate significant differences at p < 0.05 by Fisher LSD test.
Conclusions

The DFF effect on soil CO$_2$ emission followed the same trend in all studied injection depths with high emission in the first hour after distribution, and a significant reduction already after 24 hours, reaching values similar to un-amended plots after 48 hours. Comparing the emissions measured 1 hour after digestate injection, CO$_2$ flux decreased when injection depth increased, with significantly higher emission in the 10 cm treatment (median value 23.7 g CO$_2$ m$^{-2}$ h$^{-1}$) and the lowest one in the 35 cm treatment (median value 2.5 g CO$_2$ m$^{-2}$ h$^{-1}$). During maize growing season, no significant soil CO$_2$ emission differences were monitored among treatments with a median value of 0.33 g CO$_2$ m$^{-2}$ h$^{-1}$. The significantly higher cumulative CO$_2$-C soil emission during the experimental period was found for 10 cm and 25 cm treatments, with an average value of 411.8 ± 63.6 g CO$_2$-C m$^{-2}$; no significant difference was found between 35 cm and ND treatments, with an average value of 301.3 ± 49.0 g CO$_2$-C m$^{-2}$.

Digestate distribution significantly improved the maize aboveground dry biomass with an average production of 22.0 Mg ha$^{-1}$ and 16.2 Mg ha$^{-1}$ in amended and un-amended plots respectively, whereas no significant influence was exerted by digestate injection depth. Although obtained results clearly showed that soil digestate distribution has a positive effect on maize yield and the increase of injection depth reduces CO$_2$-C soil losses further studies are needed to predispose an overall CO$_2$ budget especially for deeper injection.
Chapter IX
General conclusions
During the experimental activities the greenhouse gas (GHG) emissions were evaluated from three constructed wetland plants treating urban wastewater or digestate, vegetated with five different plant species and located in two different Italian climatic conditions. Given the lack of literature on this topic for the Mediterranean Basin, further experimentation might provide more exhaustive conclusions, but from the data reported in this thesis, the following observations can be made.

In the Sicilian experimental conditions:

- GHGs emissions were influenced by season, the lower CO₂ emissions were detected in spring while the higher ones in summer and autumn whereas lower and higher CH₄ emissions were quantified in spring and autumn, respectively.
- Plant presence and different species, although not influencing CWs depuration efficiency, significantly influenced CO₂ and CH₄ emissions. As concerns CWs beds CO₂ emissions: 1) in full-scale CW significantly higher flux was monitored in vegetated areas (P. australis) than unvegetated ones; 2) in CWs pilot plant significantly higher CO₂ emissions were detected in the beds vegetated with A. donax, M. giganteus and P. australis than those with C. papyrus and C. zizanioides, which didn’t show significantly different emissions compared to the unvegetated bed. Regarding CH₄ emissions, which were only evaluated in CWs pilot plant, unvegetated bed and vegetated with M. giganteus showed significantly higher emissions than the beds vegetated with C. papyrus.
- Evaluating cumulative CO₂(eq) balance in the two years of the pilot plant trial, all vegetated beds showed positive values, with the best performance for A. donax followed by P. australis. Instead, as expected, the unvegetated bed had a net cumulative CO₂(eq) emission (5.5 kg m⁻²).
- The obtained results confirmed the active and central role of plant species used in the CW systems and underlined the need for an additional environmental impact assessment, besides the depuration efficiency one, in order to maximize the beneficial environmental effects. A. donax appeared to be the best species, in terms of environmental attitude, to be used under Sicilian Mediterranean climate conditions, followed by P. australis.

In the Veneto experimental conditions:
- *P. australis* showed a better growth performance than *A. donax*, which did not regrow in the second year, although the two species did not show significantly different CW pollution abatement.

- Plant cutting at the end of the first monitoring period determined a transitory CH$_4$ emission increase (about 8 times). During the second year the absence of vegetation in *A. donax* bed resulted in a CH$_4$ emission increase. A new *A. donax* transplanting determined, after 6 weeks, a reduction of about 80% in the average CH$_4$ emission. No significantly different CH$_4$ emissions were detected from VSSF and HSSF beds, vegetated with *P. australis*.

- CO$_2$ emissions did not show significant difference between the two seasons and CW beds.

- N$_2$O emissions were not significantly different among the three monitored beds for each season. Considering each bed in the two monitored seasons, VSSF beds did not show significant differences, whereas the HSSF one showed a significantly lower emission in the summer compared to the spring. The CW plant N$_2$O-N emissions were 1.27% and 0.87% of the CW TN removal and inlet, respectively.

- The results confirmed the central role of plant species and their management (harvest) to reduce GHGs emissions from CW systems. Opposite results from those obtained in Sicily in CW treating urban wastewater were found in Veneto region in CW treating digestate, where *P. australis* showed better growing performance than *A. donax*. No significantly different depuration performance between these macrophytes were monitored in either Sicily or Veneto.

Taking into account the digestate composition and considering it as a byproduct to be used as a resource, the effect of soil texture and preparatory tillage after digestate splash-plate spreading and digestate injection depth were evaluated in order to reduce soil CO$_2$ emission. Although results concerned data from only one year the following observations can be made:

- The digestate effect on soil CO$_2$ emission in the days after application, followed the same trend in all studied conditions with higher emissions in the first hour after distribution and a reduction already after 24 hours, reaching significantly lower values, similar to un-amended plots, after 48-72 hours.
- Considering digestate splash-plate spreading, in order to reduce soil CO\textsubscript{2} emission, results indicated clay loam soil as more suitable for digestate spreading independently of soil preparatory tillage, plowing or ripping.

- Considering digestate injection, results showed that from digestate distribution to maize harvest, the lowest CO\textsubscript{2}\textendash{}C emission of C from digestate was calculated for 35 cm injection depth (38.3%), whereas similar values were found for 10 cm (86.8%) and 25 cm (87.4%). Although the increase in injection depth reduced the CO\textsubscript{2}\textendash{}C soil losses further studies are needed to predispose an overall CO\textsubscript{2} budget.
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