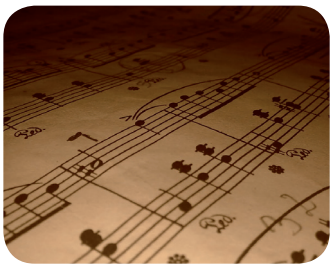

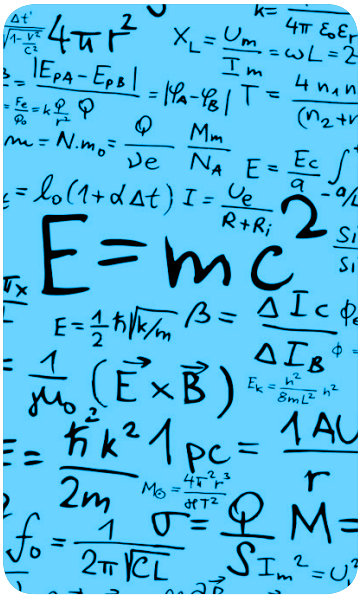

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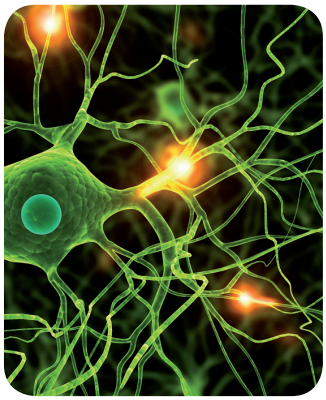
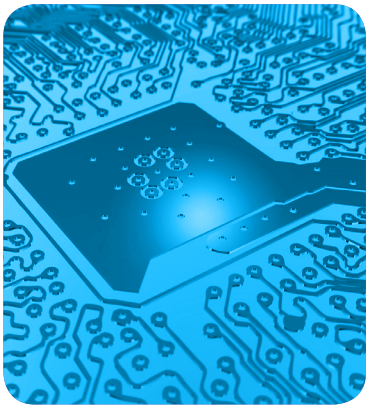
Methane dynamics in wetland sediments under anaerobic conditions

Carlos Menor Salazar





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The MSc. Student declares the authority of this master thesis
"Methane dynamics in wetland sediments under anaerobic conditions"

Sampling campaign, experiments conducted in laboratory (both methods and materials), as well as statistical analyses were conducted by his own during years 2012 and 2013 with the directorship of Salvador Sánchez-Carrillo and Isabel Herráez, and the valuable help from the Ecosystem Biogeochemistry Department (MNCN-CSIC) PhD students and technical staff, in which Salvador Sánchez-Carrillo is the Head of the Dpt.

(Photograph taken in October 2012,
inside the boat of the last fisherman in Tablas de Daimiel,
Julio Escuderos).

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The seeds of what I am, I owe it to my family. They grew me up and encouraged me to work hard every day. A quite interesting, in a scientific view too, kind of matriarchal family that I sincerely love with passion. And also I owe it to my friends. They inspired me more than I would have dreamed, and more than that, thought always in my possibilities. Dreams… they come and they leave, and this last year was especially hard, when you feel the loss of the best friend you have ever had. But always, something takes strength, as in nature, to become a new life project, and this is for you, my beloved heart.

¡Thank you all! Carlos ☺

ABSTRACT

Changes in concentrations and rates of methanogenesis, sulfate and ferric reduction were studied in sediments from a semiarid wetland (Las Tablas de Daimiel National Park) according to the main hydrogeomorphological areas: littoral, open-water areas and deep sites. Samples were taken in replicated cores (0-30 cm depth) and slurries were prepared under anaerobic conditions in order to carry out incubations at 25º C and darkness during 144 h. After 72 h and 144 h slurries reached redox potentials which favoured methanogenesis (-244 mV). After 144h of incubations range of concentrations of main variables were: CH_4 0.001-0.031 µmol / g DW; CH_3COO^- 3.5-5.5 µmol/ g DW; H_2S 0-3.3 µmol/ g DW; SO_4^{2-} 1.6-20.6 mmol / g DW; Fe^{2+} 0-0.182 µmol/ g DW; and Fe^{3+} 0.029-1.110 µmol/ g DW. Changes in methane (CH_4) concentration during incubations demonstrated that methanogenesis process was biased according to nutrient-rich areas of the wetland: higher methanogenesis rates were often obtained in nutrient-enriched sites (those located near the wetland inlet) and lower ones in nutrient-poor sites (at the end of the wetland or deep sites). Methane production did not change with depth variation. Methanogenesis was not nutrient-limiting, as acetate was accumulated rather to be consumed during experiments. Sulfate reduction seemed to be the most important degradation pathway of organic matter under anaerobic incubations. Despite of lower redox achieved during incubations, ferric reduction process was lower than those recorded in short-time incubations in other wetlands. However ferrous ion and hydrogen sulfide might exhibite some interactions that could form ferrous sulfide compounds. Our experimental study demonstrated that methanogenesis can be inhibited under elevated soil sulfate concentrations observed in Las Tablas de Daimiel. The knowledge of anaerobic processes in different wetland types is basic for a better understanding of wetland responses to future global change and their contributions to global greenhouse gas emissions.

Keywords: *methanogenesis, sulfate reduction, anaerobic processes, semiarid wetland, Las Tablas de Daimiel National Park*

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ABBREVIATIONS

TDNP: Tablas de Daimiel National Park
GHG: Greenhouse Gas
GC/FID: Flame Ionization Detector Gas Cromatography
IC: Ion Cromatography
SP: Spectrophotometer (UV/Vis)
CHNS/O: Elemental Analyzer of Carbon, Hydrogen and Nitrogen

TERMS AND DEFINITIONS

Tablazo and/or *Tablas*: in Spanish is a wide open water area free of emergent macrophytes in wetlands area. Usually is found in floodplains where soft flat depressions are a common landscape and are covered by submerged macrophytes like Charophytes.

Slurry: is a prepared thin mud consisting on a mixture of sediment and distilled water, often used as a convenient way of handling solids in bulk. Slurries behave in some ways like thick fluids, enabling continuous stirring and mixing during experimental incubation.

Source/sink: in ecology and biogeochemistry (i.e. in carbon or methane emissions) an ecosystem is source when the budget between production and consumption is positive, being sink when this balance is negative (Le Mer and Roger, 2001).

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1. INTRODUCTION AND BACKGROUND

Methane (CH_4) is an atmospheric trace gas that play important role in the earth's energy balance, out of proportion to their concentrations. Although methane's concentration in the atmosphere is much lower than CO_2, it more effectively absorbs infrared radiation and therefore contributes significantly to the warming of the planet due to the greenhouse effect (Barlett and Harriss, 1993). Rodhe (1990) and more recently Prather et al (2001) estimated that methane contributes 15-20% to the anthropogenic greenhouse effects (CO_2 contributes about 60%).

Wetlands are not only ecologically but also economically important systems. Being in the transition between terrestrial and aquatic ecosystems, wetlands are buffers for terrestrial runoff, thereby preventing eutrophication of inland as well as coastal waters, as inputs of nutrients and fast recycling due to active aerobes and anaerobes makes these systems highly productive (Bodelier and Dedysh, 2013). The interface of oxic–anoxic conditions, often created by macrophyte roots and enhanced by the hydrological regime, promotes the coexistence and activity of aerobic/anaerobic microbial communities (Mitsch and Gosselink, 2007). However, wetlands globally are under high pressure due to anthropogenic activities including climate change. Changes of land-use and altered hydrology due to climate change will lead to disturbance and loss of these habitats. Nonetheless, the diversity and functioning of microbial communities in wetland systems is highly underexplored yet in comparison to soils and aquatic ecosystems (Bodelier and Dedysh, 2013).

Wetlands represent a large carbon reservoir (37%-58% of the global terrestrial carbon in spite of covering only between 4% and 9% of the global surface area (Matthews and Fung, 1987; Bolin and Sukumar, 2000; Bloom et al., 2009) that is a potentially important source of CO_2 if warming occurs. According to the International Panel on Climate Change (IPCC, 2007) wetlands are a significant source of atmospheric methane (CH_4), contributing 20-39% of the global CH_4 emissions to the atmosphere on a yearly basis (Fung et al., 1991; Roulet et al., 1992; Mitsch and Gosselink, 2007). The average of net methane annual emissions from wetlands is estimated to be 145 Tg/yr, ranging between 115-237 Tg/yr (Fung et al., 1991; Lilieveld et al., 1998; and Walter et al., 2001; Gedney and Cox, 2003a, b; Chowdhury and Dick, 2013), being the interannual variability of CH_4 emissions about ± 12 Tg/yr (Bousquet et al., 2006). The wetland oxidation has been estimated to be 40-70% of gross CH_4 production (100-400 Tg/yr; Reeburgh et al., 1993; King, 1996).

Global change and wetland metabolism are known to have interactions often acting as positive feedback for future warming. However, there is still uncertainty of how the interaction of various factors exacerbate or mitigate the radiative forcing due to an increase in GHG (Megonigal et al., 2004): either non-additive (*not having a numerical value, sum of the values of the different parts*) or counterintuitive (*contrary what seems intuitively right or correct but with desirable outcomes*). Therefore, wetlands may play an important role in current and future climate scenarios, either as a source or sink for CH_4 and other greenhouse gases.

Role of wetlands in terrestrial carbon cycling with focus in methane dynamics

The role of wetlands in the terrestrial carbon cycling is particularly complex mainly due to the fact that in a very thin layer of wetland soils occur almost simultaneously methane production, methane oxidation, sulfate reduction and ammonium oxidation (anammox; Roehm, 2005; Luesken et al., 2011). Many global and regional reviews on CH_4 emissions from wetland environments have been carried out according with different approaches (IPCC, 2007): (1) flux extrapolation (Aselmann and Crutzen, 1989; Matthews and Fung, 1987; Barlett and Harris, 1993), (2) process modeling (Segers, 1998) and (3) inverse modeling (Bousquet et al., 2006). Also modeling and predicting emission at regional or wider scales have been assessed in wetlands (Roehm, 2005; Bridgham et al., 2006; Bastviken et al., 2011). **Table 1.1** offers a summary of reviews on CH_4 emissions (EPA, 2010).

Table 1.1. Summary of estimated wetland CH_4 fluxes by different approaches (Tg CH_4/Year). (EPA, 2010)

Approach	Northern/Bogs	Tropical/Swamps	Total
Flux extrapolation	31–48[a] avg = 38 (37%)	49–80 avg = 65 (63%)	80–115 sum of avgs = 103; n=4
Process modelling	20–72[b] avg = 44 (31%)	41–133 avg = 90 (64%)	92–156 sum of avgs = 134 n=8(bogs); 5 (swamps)
Inverse modelling	21–47 avg = 36 (20%)	81–206 avg = 144 (78%)	145–237 sum of avgs = 180
Current best guess (process and inverse modelling since 2004)	24–72 avg = 42.7 (25%) std. dev. = 16.6; n = 10	81–206 avg = 127.6 (75%) std. dev. = 44.0; n = 8	170.3 range = 105–278 by summing minima and maxima

[a] For flux extrapolation, temperate emissions are split equally between bogs and swamps. Values in parentheses indicate percentage contribution to wetland total emissions.

[b] Walter et al. (2001) estimates excluded.

For example, Barlett and Harriss (1993, **Figure 1.1**), compared global CH_4 emissions estimates from different wetlands-type based on latitude and climate zones by using emission season estimates from Matthews and Fung (1987). In this Figure, high latitudes account to 33.60 Tg CH_4/yr, temperate 5.45 Tg CH_4/yr and tropical 65.60 Tg CH_4/yr.

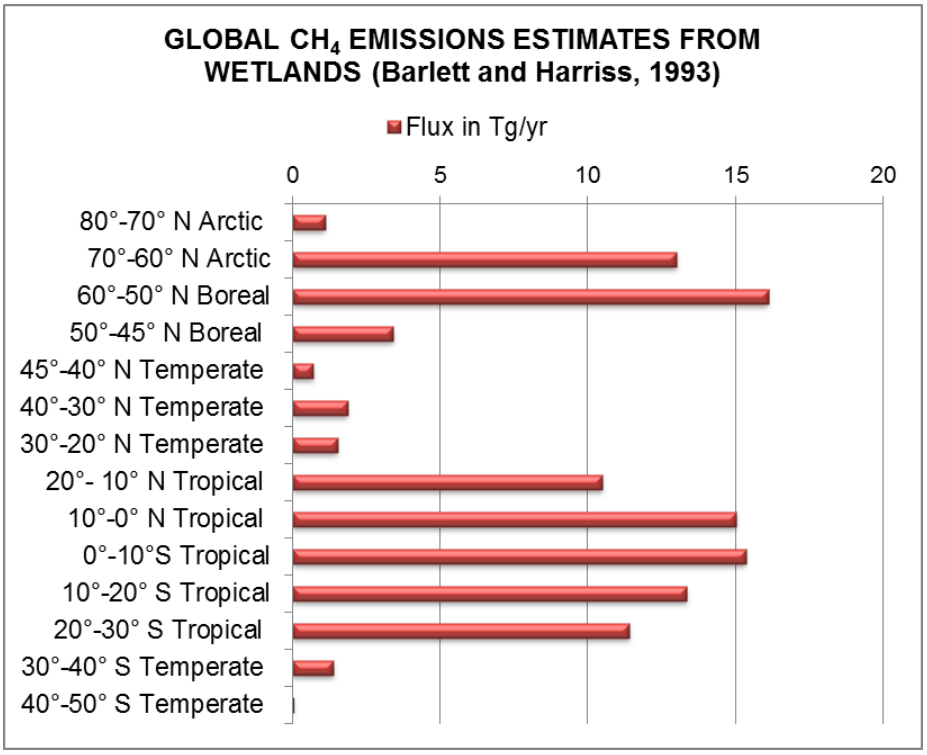


Figure 1.1. Global CH_4 emissions estimates from wetlands (modified from Barlett and Harriss, 1993).

Differences between habitat types are likely to arise from a variety of factors, including differences in organic inputs, water depths and rates of flow, and exposure to wind-driven mixing. Seasonal differences (not shown here) suggest the importance of CH_4 release by bubbling which, even though sporadic, can emit large amounts of gas, with wind, depth (inversely) and solar radiation controlling bubbling events (Bartlett and Harriss, 1993; Keller and Stallard, 1994).

Methane cycling in freshwater wetlands: biogeochemical overview

Methanogenesis is the formation of methane by microbes known as methanogens. Organisms capable of producing methane have been identified only from the domain Archaea, a group phylogenetically distinct from both eukaryotes and bacteria, although many live in close association with anaerobic bacteria (Chowdhury and Dick, 2013).

The production of methane is an important and widespread form of microbial metabolism. In most environments, it is the final step in the decomposition of organic matter (Megonigal et al., 2004; **Table 1.2**).

Table 1.2. Potential redox (Eh) and free energy of activation (AG) in reduction processes in anaerobic conditions (Megonigal et al., 2004).

Reaction (at pH 7 and 25ºC)	**Eh (mV)**	**AG (Kcal mol/e$^-$)**
Reduction of O_2	812	-29,9
Reduction of NO_3^-	747	-28,4
Reduction of Mn^{4+} to Mn^{2+}	526	-23,3
Reduction of Fe^{3+} to Fe^{2+}	-47	-10,1
Reduction of SO_4^{2-} to H_2S	-221	-5,9
Reduction of CO_2 to CH_4	-244	-5,6

Methanogens are obligate anaerobes that can grow autotrophically, using CO_2 as an electron acceptor (net energy yield of -134 kJ/mol), and also heterotrophically, using organic compounds as energy source (net energy yield of -130 kJ/mol; Le Mer and Roger, 2001). Both counts for methane production but results in a yield approximately 10% of the aerobic respiration energy yield. During the decay process, electron acceptors (such as oxygen, nitrate, ferric iron and sulfate) become depleted, while hydrogen (H_2) and carbon dioxide accumulate. Light organics produced by fermentation (e.g.: acetate) also are known to accumulate (Hines et al., 2001). At advanced stages of decomposition under anaerobic conditions, all electron acceptors become depleted except carbon dioxide. Carbon dioxide is a product of most catabolic processes, so it is not depleted like other potential electron acceptors. Only methanogenesis and fermentation can occur in the absence of electron acceptors other than carbon. Methanogenesis effectively removes the semi-final products of decay: hydrogen, small organics, and carbon dioxide. This occurs at potential redox below -244 mV (Megonigal et al., 2004; **Table 1.2**).

The two best described pathways involve the biogenic methanogenesis in nature is primarily derived from acetate fermentation (acetoclastic methanogenesis) and from H_2 oxidation coupled with CO_2 reduction (Oremland, 1998):

1) Hydrogenotrophic or CO_2/H_2 reduction or H_2-dependent methanogenesis (about the 73% of methanogenic species; Garcia et al., 2000).

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$$

2) Acetotrophic or acetate fermentation or acetoclastic methanogenesis (less than the 10% of methanogenic species; Garcia et al., 2000).

$$CH_3COOH \rightarrow CO_2 + CH_4$$

It is usually recognized that acetotrophic methanogenesis is dominant in freshwater ecosystems, while hydrogenotrophic methanogenesis is dominant in marine systems (Whiticar, 1999). Küsel and Drake (1994) found that flooded soils accumulated acetate for several months without starting the methanogenesis, suggesting that acetoclastic methanogens grow more slowly than those H_2/CO_2 reducers (Shannon and White, 1996). **Table 1.3** shows the microbial processes involved with acetate as electron donor and the ΔG^o (kJ) per reaction.

Table 1.3. Microbial processes involved with acetate as electron donor.

	Reaction	**AG^o (kJ per reaction)**	**Kinetically**
$CH_3COO^- + 8\ Fe^{3+} + 4\ H_2O \rightarrow 8\ Fe^{2+} + 9\ H^+ + 2\ HCO_3^-$	*Iron reduction*	-808,6	↑+ *favourable*
$CH_3COO^- + 8/5\ NO_3^- + 3/5\ H^+ \rightarrow 2\ HCO_3^- + 4/5\ N_2 + 4/5\ H_2O$	*Denitrification*	-791,9	
$CH_3COO^- + SO_4^{2-} \rightarrow HS^- + 2\ HCO_3^-$	*Sulfate reduction*	-47,6	↓ – *favourable*
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	*Methanogenesis*	-31	

Values calculated with data from Sober (1970) and Thauer et al., (1977)

Production and emission of methane in wetlands depend on numerous physical, chemical and biological properties of soils and plant community structure (Wang et al., 1993; Bubier et al., 1993; Yavitt et al., 1997; Moore et al., 1998; **Figure 1.2**). Methanogenesis is often more sensitive to temperature than other biological processes, which typically double in rate with a 10 ºC increase (Segers, 1998), being severely suppressed under lower temperatures (Megonigal et al., 2004). In fact, the time required for methanogen competitors to deplete the pool of alternative electron acceptors decreases with increasing temperature (Van Hulzen et al., 1999). Moreover, methanogenesis is most active when the consumption rate of alternative electron acceptors exceeds their supply rate (Megonigal et al., 2004) although there are some exceptions (Metje and Frenzel, 2005).

In wetlands, another important factor that directly affects both production and emission of methane is hydrology. Although inundation in wetlands promotes anoxic conditions and therefore methanogenesis, it is not clear yet the effects of hydrological fluctuations on methane emissions (Reddy and DeLaune, 2008).

The release of methane from aquatic ecosystems can be done through different pathways: as bubble ebullition from sediments, as dissolved methane fluxes, and by aerenquima transport in emergent aquatic macrophytes (Le Mer and Roger, 2001; Bastviken et al., 2004, 2010, **Figure 1.2**).

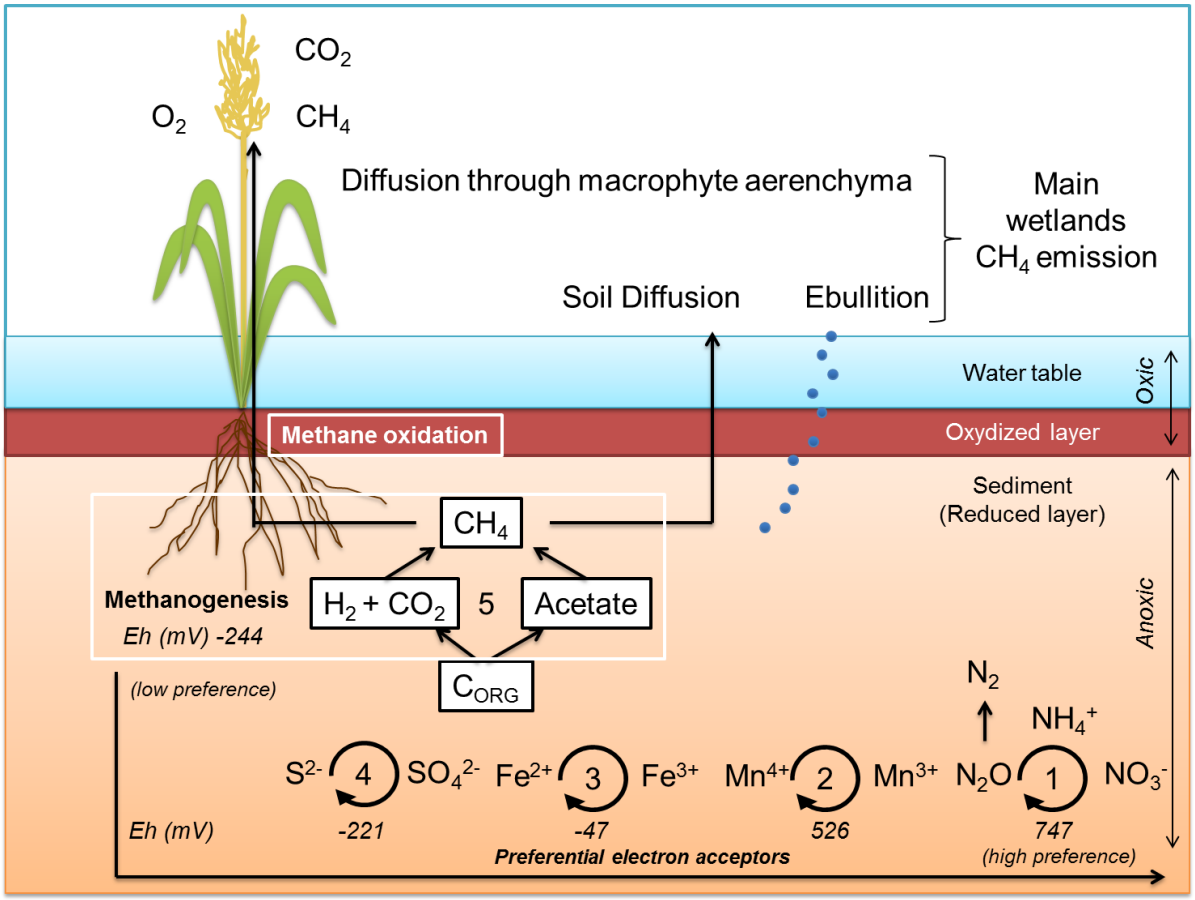


Figure 1.2. Production, consumption and transfer of methane in wetlands (modified from Le Mer and Roger, 2001 and Bastviken, 2010).

Methane oxidation (also known as "reverse methanogenesis") can occur under aerobic and anaerobic conditions. Reverse methanogenesis is a pathway that can oxidize methane to carbon dioxide. This is a natural process occurring in many aerobic and anaerobic species. In the case of a bacteria oxidizing methane without the presence of O_2, the reaction must be coupled with another to push the reaction forward.

There are two pathways suspected in the oxidization of methane in anaerobic conditions: (i) AOM or "anaerobic oxidation of methane" requires two bacteria working together to oxidize methane, and (ii) ANME, which stands for "anaerobic methanotroph" are species of bacteria that can oxidize methane with the help of other bacteria in the same environment. The oxidation of methane within the ANME bacteria leaves electrons free floating where they must be transferred to different bacteria to be used. In this circumstance, the electrons are coupled to a reaction utilizing the reduction of sulfates (Zehnder and Brock, 1980; Caldwell et al., 2008).

Partial oxidation of CH_4 has been reported in marine anoxic sediments and in submerged soils. Methane is diffused upward from deeper sediments horizons and then consumed by prokaryotes by an undefined process in which sulfate is known to act as the terminal oxidant (Knittel and Boetius, 2009). This process is estimated to consume CH_4 equivalent to 5-20% of the net atmospheric flux (20-100 Mtn yr^{-1}; Valentine and Reeburgh, 2000). This mechanism is run by sulfate-dependent methane oxidation (SDMO) but only under sulfate-limiting conditions (Muyzer and Stams, 2008). Segers (1998) suggests that anaerobic methane oxidation in freshwater systems could be possible from sulfate concentrations around 1 mM, which is relatively high for natural freshwater wetlands.

Hoehler et al. (1994) suggested a consortium of methanogens and sulfate reducers performing the reverse methanogenesis, since it is biochemically feasible for methanogens and sulfate reducers the net CH_4 oxidation and hydrogen syntrophy. In marine anaerobic environments hydrogen appears as a competitive substrate and its concentration represents a dynamic steady state which is indicative of the dominant terminal electron-accepting process. Under sufficiently low H_2, methanogens reverse their metabolism and mediate the net reversal of methanogenesis (acting as methane oxidizers) using water as the terminal electron acceptor. Furthermore, H_2 is removed efficiently and maintained at low concentrations by sulfate reducers operating in a syntrophic association with the methanogens (Hoehler and Alperin, 1996). The sulfate reducers are more efficient using H_2 as an electron donor, thereby creating conditions that thermodynamically favor CH_4 oxidation under anaerobic conditions. The net reaction of the syntrophic association yields approximately 25 kJ/mol CH_4 oxidized (Knab et al., 2008).

$$CH_4 + 2H_2O \rightarrow CO_2 + 4H_2 \text{ (MO)} \quad (1)$$

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \text{ (SBR)} \quad (2)$$

$$SO_4^{2-} + CH_4 \rightarrow HCO_3^- + HS^- + H_2O \text{ (Net)} \quad (3)$$

However, a major question about the mechanism is the finding that many methanogens (*Methanosarcinales*) are unable to use H_2 during methanogenesis, and tend to grow from methylated compounds and acetate instead (Hinrichs et al., 1999). They operate by the formation of acetic acid and H_2 from CH_4 by methane-oxidizing archaea with subsequent H_2 and acetic acid consumption by sulfate reducers:

$$2CH_4 + 2H_2O \rightarrow CH_3COOH + 4H_2 \text{ (MO)} \quad (4)$$

$$SO_4^{2-} + 4H_2 + H^+ \rightarrow HS^- + 4H_2O \text{ (SBR)} \quad (5)$$

$$CH_3COOH + SO_4^{2-} \rightarrow 2HCO_3^- + HS^- + H^+ \text{ (SBR)} \quad (6)$$

$$CH_4 + 2SO_4^{2-} \rightarrow 2HCO_3^- + HS^- + 2H_2O \text{ (Net)} \quad (7)$$

The coupling of two CH_4 molecules to form acetic acid allows for energy conservation from the net process. The net reaction (Eq. 7) provides twice as much free energy as the mechanism of reverse methanogenesis (Eq. 3). Effectively, the small amount of energy available in methanogenic metabolism effectively forces the bacteria into symbiotic relationships (Schink, 1997).

Finally, although out of our scope, recent findings have shown others microbial oxidations of methane different than those with oxygen or sulfate are possible. It has been cited an anaerobic oxidation of methane coupled to denitrification of nitrate (Raghoebarsing et al. 2006), and a simultaneous nitrite-dependent anaerobic methane and ammonium oxidation process (Luesken et al., 2011). These findings increase our knowledge on the methane cycling but also shows the extreme complexity involved in the carbon cycle in natural environments.

2. OBJECTIVES

The main objective of this study was the assessment of methane dynamics in sediment wetlands in a Spanish semi-arid wetland (Las Tablas de Daimiel National Park, Central Spain). To achieve this goal, our study faced with these specific aims:

(1) To assess the methane production/consumption rates in sediments after sediment exposition to anoxic conditions
(2) To assess the main sediment layer (depth) contributing to methane production/consumption.
(3) To assess the methanogenesis dependence of acetate availability in wetland sediments.
(4) To assess the methane dynamics relationship with sulfate reduction.
(5) To assess the main factors controlling the methane dynamics in this wetland site.

For these purposes a laboratory study has been performed consisting in anaerobic incubations (144 h) of duplicated sediment cores (prepared slurries) from different wetland type-habitats taken into account the different dynamics of microbial activities by soil depths (0-10, 10-20 and 20-30 cm).

The main questions to be answered in this study were:

- *Do the methane production rates follow a linear trend over time?*
- *Does the methane production increase with sediment depth?*
- *Does the methane consumption increase in surface sediments?*
- *Which is more important in the methane production dynamics: the availability of acetate or other constraints?*
- *Are the methane cycling coupled to sulfate reduction (consortium of methanogenic bacteria and sulfate reducers)?*
- *What factors (time exposure to anoxic conditions, plant cover, carbon content, hydroperiod) are the main controlling methane cycling in this wetland?*

3. MATERIAL AND METHODS

3.1. Study Area

Las Tablas de Daimiel National Park (TDNP hereafter) is located at Central Spain in the Castilla-La Mancha Autonomous Community (39º08'N, 3º43'W; **Figure 3.1**). The climate of the basin is semiarid, with an average annual rainfall of 300-500 mm and an average annual temperature ranging 14-15ºC (Pérez-González and Sanz-Donaire, 1998).

Until the 1970s, wetland occurrence was due to natural flooding in the extended alluvial plain of both the Gigüela and the Guadiana Rivers, the latter flowing from the Ojos del Guadiana, a groundwater source area located around 10 km East of TDNP. The floodplain wetland was also the natural groundwater discharge zone of the Llanura Manchega Occidental aquifer (23 Aquifer or 04.04 Hydrogeologic Unit according to the current official nomenclature of the Geological Survey of Spain–IGME) as the groundwater table was close to the surface. Therefore, TDNP inundation was supported by numerous groundwater surges along the wetland known as "Ojos" or "Ojillos" ('eye's or 'little eyes'). Furthermore, the wetland´s flooded area was also maintained by human buildings, such as small water-mill dams which helped to retain water in TDNP. At least 15 water-mill dams have been identified along the Gigüela and Guadiana Rivers (Álvarez-Cobelas et al., 1996). Thus, the waterscape of TDNP appeared as the result of both beneficial natural flooding and human-induced inundation in an area where rainfall is scarce (Sánchez-Carrillo and Angeler, 2010).

At the present moment, the potentially flooded area of TDNP is of 15.87 km^2. At the end of TDNP –southwest area– the Puente Navarro dam, a domed concrete dam, controls the water storage in the wetland acting as a reservoir (Álvarez-Cobelas et al. 1996).

European cut-sedge (*Cladium mariscus*) was the dominant population of emergent macrophytes, accompanied by reed (*Phragmites australis*) and cattail (*Typha domingensis*) restricted to littoral areas (Cirujano et al., 2010). TDNP represented the more important cut-sedge cover in Western Europe (Álvarez-Cobelas and Cirujano 1996) but, at present, this macrophyte is in regression due to wetland degradation (Álvarez-Cobelas et al. 2001). Wetland richness was also supplied by numerous vertebrate and invertebrate taxa, which regrettably disappeared or are undergoing a severe number reduction (Álvarez-Cobelas and Cirujano 1996).

International recognition of TDNP and its protected status appeared as a consequence of the habitat importance for waterfowl. Most representative and profuse waterfowl in TDNP are the mallard (*Anas platyrhynchos*), the common teal (*Anas crecca*) and the red-crested pochard (*Netta rufina*), the emblematic waterfowl in La Mancha Húmeda wetlands (Sánchez-Carrillo and Angeler, 2010).

After a several drought period (2005-2010), where the peat fire threatened to destroy the entire wetland, currently the ecosystem is experiencing one of its best hydrological cycles (2011-2013; **Figure 3.1**): high inundation, including summer months, few episodes of contamination, clear waters, recovery of waterfowl populations, etc., although its environmental recovery remains a matter of concern.

Figure 3.1. Aerial view (from Northeast to Southwest) of Las Tablas de Daimiel National Park in 2012. The inlet area of Guadiana river is shown in the lower area of the picture while the main water flow pathway occurs from right to left. (Photography courtesy of S. Cirujano).

3.2. Field Sampling

A total number of 8 sites for sediment sampling were selected according to different environmental characteristics of the wetland (**Figure 3.2**):

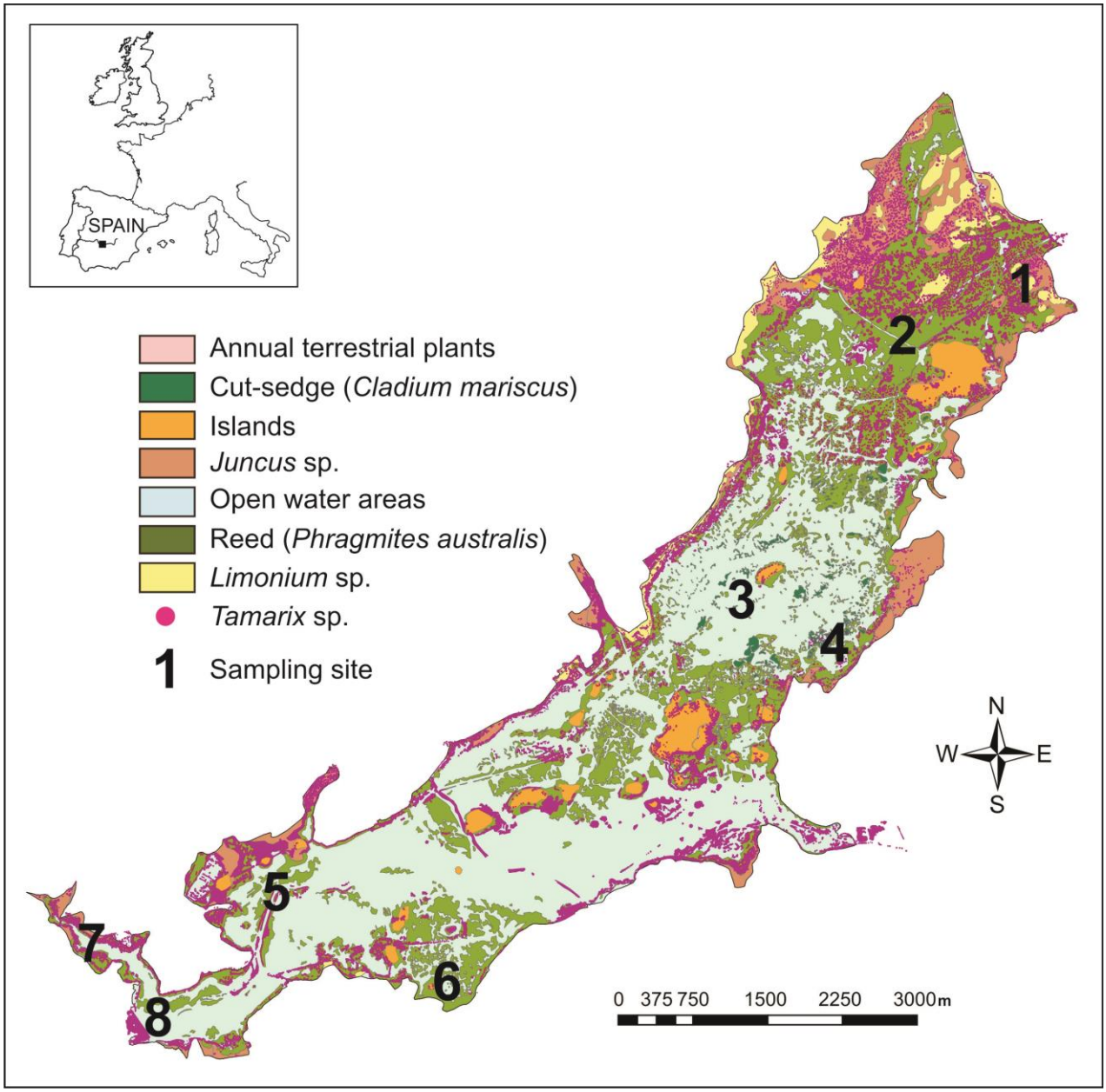


Figure 3.2. Vegetation map of Las Tablas de Daimiel National Park in 2010 (map courtesy from Santos Cirujano). Sampling site numbers stand for local names: 1-Algeciras (littoral zone), 2-Tabla de Algeciras, 3-Tablazo, 4-Hides (littoral), 5-Almochinares, 6-Los Corrales, 7-Cachón de la Leona, 8-Puente Navarro.

1) Littoral zones (four sites: 1, 4, 6, 7): very shallow environments, only inundated during high water-level episodes (1-3 month per year except in drought cycles), covered by emergent macrophytes (*Phragmites australis*), and abundant microbial mats.

2) Tablas zones (two sites: 2, 3): open water shallow sites (50-100 cm) frequently inundated (3-12 month per year except during droughts), and covered by submerged macrophyte patchs (*Chara vulgaris* and *C. hispida*).

3) Deep zones (two sites: 5, 8): very frequently inundated (more than 6 month per year except in drought periods) with water depth oscillating from 2-4.5 m; macrophyte plants are restricted to littoral zones and phytoplanktonic blooms may occur during late summer.

Replicates sediment cores (6 cm diameter) were collected by hand in October 2012. Immediately after collection, cores were sliced at 10 cm intervals until 20 or 30 cm (0-10 cm, 10-20 cm and 20-30 cm) sediment depth, according to Van der Nat et al. (1997). In *Site-8* an Ekman dredge was used due to the surface sediment hardness (only samples of 0-10 cm were collected). All sediment samples were immediately transferred to 1/2 L glass jars, filled with water of each sampling site until the top and then sealed with aluminum caps (that allow to keep without any bubbles). All samples were conserved below 4° C in darkness during the trip to the laboratory. Due to laboratory constrains, samples were kept under refrigeration (<4º C) for 2 months until experimental incubations started. As pointed out by Miller et al. (1998), the lower temperature maintained the microbial activity as negligible.

3.3. Slurry Preparation and Incubation Procedures

Slurries were prepared for each sediment subsample (0-10 cm, 10-20 cm, 20-30 cm) using fresh homogenized sediment and distilled water (which was previously bubbled with N_2 during 30 minutes) until obtain an average density of varying 0.04-0.08 g/cm^3 (e.g. 15 g of soil in 250 ml of water; **Figure 3.3**). Since the anaerobic conditions must be maintained throughout processes, all these operations were conducted on a hermetic glove-box with continuous N_2 flux. Once slurries were prepared aliquots were immediately separated in headspace vials capped with septum rubber stoppers (Dionex® septums) in order to proceed with incubations. Although vials must be shaken until equilibrium is reached between the headspace gas and dissolved gas, very gentle stirring was used in order to minimize the disruption of microbial consortia (Dannenberg et al., 1997; Metje and

Frenzel, 2005). To allow a better handling of samples, the experiments were conducted in 3 batch series: (1st) for methane (gas sample) and acetate concentration changes (liquid sample), 555 incubations by triplicate at time 0-24-48-72-144 hrs; (2nd) for ferrous-ferric ions and hydrogen sulfide concentration changes (both liquid samples), 222 incubations by duplicate at time 0-72-144 hrs; and (3rd) for sulfate concentration changes (liquid samples) 148 incubations by duplicate at time 0-144 hrs. At the beginning and end of each incubation, redox potential and dissolved oxygen were measured using a HACH lange HQ40d multiparameter. All experiments were conducted in an incubation chamber at 25º C in darkness. Data were normalized by sediment dry-weight (DW) after drying at 110º C of each sediment fractions during 48 h and corrected by the density of slurries. Mean soil bulk density was calculated, being 0.8 g/cm^3.

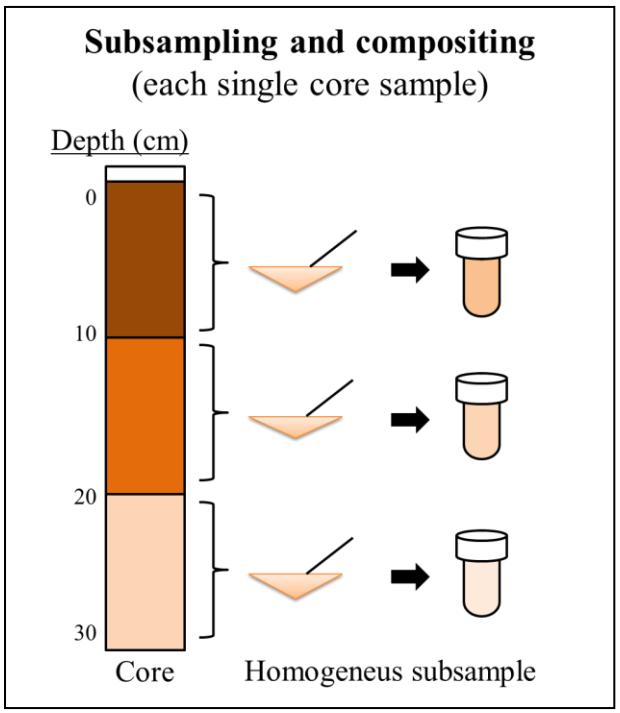


Figure 3.3. Diagram of subsampling method used in this study.

3.4. Incubation Experiment Details

3.4.1. Methane

Due to the interest to reproduce in laboratory conditions incubating sediment slurries and monitoring headspace methane volume concentrations over time, the experiment addressed the potential rates in a broad sense without addition of specific substrates for methanogenesis (Segers, 1998; Nguyen et al., 2010).

As previously cited, incubations were conducted using 60 ml of slurry by triplicate at 24 h, 48 h, 72 h and 144h and the initial concentration measured. After incubation time, from each 60 ml-vial 1 ml-gas volume was extracted using a gas-tight syringe (GC-Hamilton syringe), collected thereafter in an exetainer vial (Exetainer®, Labco, UK) and after analyzed by gas chromatography (GC-FID) using an Agilent 6890N with He as a carrier gas, detector temperature column set to 120º C and temperature injector to 150ºC. The column used was HP-PLOT/Q (30 m length × 0.320 mm ID and 20.00 µm film). Gas chromatograph was daily calibrated using CH_4 standards (Conrad et al., 1989; Upstill-Goddard et al., 1990). The calibration curve used for CH_4 estimations is shown in **Figure 3.4.** After normalization, methane production or consumption results are provided both as concentration and rates in µmol CH_4 / g DW/ h.

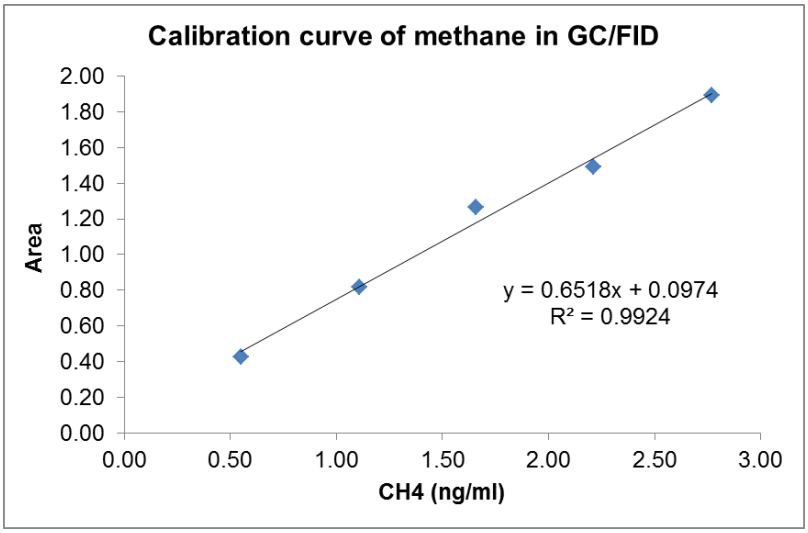


Figure 3.4. Calibration curve used for methane analyses in GC/FID.

A negative correlation between redox potential and CH_4 production has been often observed in the absence of O_2, and leading to hypothesized that:

1) Slurry incubations did not achieve good potential redox due to laboratory manipulation.
2) In some cases, it probably reflects competition between methanogens and their competitors for reductants, not a physiological requirement for a certain redox potential (Megonigal et al., 2004).
3) Related with the above statement, reverse methanogenesis has been for decades an underappreciated aspect of methane cycling but nowadays rising evidence (Smemo and Yavitt, 2006, 2011). This process may be also affecting the CH_4 cycle here studied.

In absence of conducting aerobic slurries in parallel and in completing the information given by other reduction processes, redox potential is a good indicator of changes in the slurry environment that directly affects to understand the biogeochemical processes involved.

3.4.2. Acetate (CH_3COO^-)

Changes in acetate concentration were measured in the same incubations used for methane experiments. After the incubation time was over, an aliquot of 25 ml was collected from each vial with a syringe, centrifuged (8.000 x g) during 5-10 minutes and the supernatant immediately filtered with a 0.25 µm pore size filter (Olim Peak ® Polyethersulfone-PES). Acetate was measured by Ion Cromatograph using 1 ml of this sample in a Dionex 12000 with an AS14 4-mm ion exclusion column with suppression and with a sodium carbonate eluent. Acetate standards were prepared in the laboratory from sodium acetate. Values were given in µM of acetate.

3.4.3 Ferrous Ion (Fe^{2+}) and ferric Ion (Fe^{3+})

After the incubation period was over, in the anaerobic glove-box to avoid by oxygen contamination a fast precipitation of Fe (III) oxyhydroxides and a decrease of the dissolved reduced Fe concentration, 4 ml of liquid sample was collected and filtered in 0.45 µm pore size filters. Fe^{2+} was measured by spectrophotometry using the Ferrozine method (Stookey, 1970; later modified by Viollier et al., 2000). The calibration curve used for Fe^{2+} and Fe^{3+} is shown in **Figure 3.5.**

The ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p*'-disulfonic acid) is a reagent which reacts with Fe^{2+} to form stable magenta complex species:

$$\text{Ferrozine} + Fe^{2+} \rightarrow \text{Ferrous ferrozine (magenta complex)} \rightarrow Fe^{3+}$$

Reagents:

A. Ferrozine (MW: 492.47 g mol^{-1}, 97%, Aldrich #16,060-1): 10^{-2} mol/l was prepared in an *ammonium acetate* (CH_3COONH_4, Aldrich #37,233-1, 99.999%) solution of 10^{-1} mol/l.

B. Reducing agent − *hydroxylamine hydrochloride* ($H_2NOH.HCl$, 99.9999%, Aldrich #37, 992-1): 1.4 mol/l prepared in a solution of analytical grade hydrochloric acid 2 mol/l.

C. Buffer − *ammonium acetate*: a 10 mol/l solution adjusted to pH 9.5 with a solution of *ammonium hydroxide* (28‑30%, NH_4OH, JT Baker #9721-02).

Standards were prepared from a 1000 µg ml^{-1} Fe(III) stock solution (1.786×10^{-2} mol/l of $FeCl_3$ in HCl 10^{-2}mol/l) diluted with deionized water.

Sample analysis: Firstly, 4 ml was pipetted to an opened Hach-vial and 400 µl of reagent A was added. Absorbance was measured at 562 nm, giving values for A_1 (ferrous iron). Secondly, 3.2 ml of sample + reagent A was pipetted to another opened Hach-vial adding 600 µl of reagent B. After 10 minutes for the reaction occurrence, 200 µl of reagent C was added and automatically the complex-magenta appeared and the absorbance was measured at 562 nm (yields, between pH 4 and 9, a molar absorption coefficient close to 30,000 l mol^{-1} cm^{-1}), giving values for A_2 (ferric iron). Using the equations by the simple linear system of (Eq. 1) and (Eq. 2) were solved for the Fe (II) and Fe (III) concentrations:

Calculations:

$$C_{Fe(II)} = \frac{A_1 \varepsilon_{Fe(II)}\, l\alpha - A_2 \varepsilon_{Fe(III)}\, l}{\varepsilon_{Fe(II)}\, l\alpha(\varepsilon_{Fe(II)}\, l - \varepsilon_{Fe(III)}\, l)} \qquad \text{(Eq. 1)}$$

$$C_{Fe(III)} = \frac{A_2 - A_1 \alpha}{\alpha(\varepsilon_{Fe(II)}\, l - \varepsilon_{Fe(III)}\, l)} \qquad \text{(Eq. 2)}$$

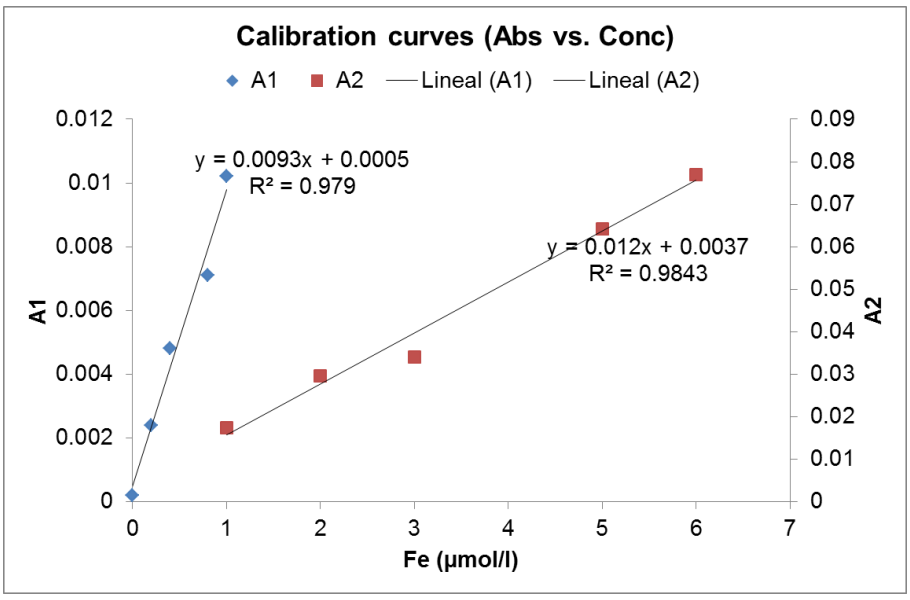


Figure 3.5. Calibration curves used for ferrous and ferric analyses using the ferrozine method.

3.4.4. Hydrogen Sulfide (H_2S)

After the incubation period finished, 4 ml of liquid sample was collected and filtered in 0.45 µm pore size filters. 0.5 ml of zinc-acetate solution was added in order to fix the compound. Later it was carried a iodometry (thiosulfate titration) and finally dissolved hydrogen sulfide was measured then by spectrophometry at 670 nm following Cline (1969) and Hayes et al. (2006).

Reagents:

A. Zinc acetate solution (0.05 M): 1.1 g of $Zn(CH_3COO)_2$ in 100 ml of distilled water.

B. Diamine mixed solution: 4.0 g of $FeCl_3$ + 1.6 g N,N-dimetil-p-fenilen sulfate diamine ($C_8H_{12}N_{12}H_2O_4S$; MW: 234,28 g mol^{-1}) in 100 ml of HCl (6 N).

C. Sulfur solution (patron): 0.5 g of sodium sulfur ($Na_2S.9H_2O$) in 250 ml distilled water and bubbled with N_2.

D. Sulfuric acid solution (1:1, v/v)

E. Indicator: 1 g of soluble starch in 100 ml of distilled water. Drops of chloroform were later added.

F. Sodium thiosulfate solution (0.01 N): 2.48 g of $Na_2S_2O_3.5H_2O$ in 1000 ml of distilled water.

G. Potassium iodate solution (0.01 N): 0.3567 g of KIO_3, previously oven-dried at 105ºC one hour, in 1000 ml of distilled water.

H. Potassium iodide: 20 g of KI in 100 ml of distilled water.

Results were given accordingly to the equation: $H_2S\ (\mu mol/l) = 2.388 - (ABS_M - ABS_B) / p$

Where ABS_M and ABS_B correspond to the absorbance of the sample and blank, respectively; p is the curve slope, calculated during the process and the constant number 2.388 is a dilution factor of the sum of volumes added: $Zn(Ac)_2$ + 4 ml of sample + H_2O (O_2-free) + diamine solution, divided by 4.

3.4.5. Sulfate (SO_4^{2-})

Slurries for sulfate incubations were prepared by duplicate with 75 g of fresh sediment in a volume of 500 ml of distilled water (N_2-bubbled during 30 min). 100 ml of the prepared slurry were incubated in glass BOD bottles with caps. After the incubation period was over, 50 mg of a soluble salt monopotassium phosphate KH_2PO_4 (1 M) was added for sulfate extraction (Tabatabai, 1996).

After agitation (1 h at 120 r/min) and filtration (Wathman 42), firstly sulfate was precipitated with barium chloride, secondly the sample was subjected to iodometric titration with CrO_4^{2-} and, finally measured by spectrophotometry (420 nm; APHA 1985:426B):

$$CrO_4^{2-} + Ba^{2+} + SO_4^{2-} \leftrightarrow SO_4Ba + CrO_4^{2-}$$

3.4.6. Carbon and Nitrogen

Total C and N sediment contents were determined with a Perkin-Elmer 2400 Series 2 CNHS/O elemental analyzer. Two subsamples were used for each analysis and further subsamples were analyzed if results did not fit with the estimated error interval.

3.5. Statistical Analysis

All variables were converted to changes in concentration respect to the initial values and to rates (production or consumption by hour of incubation). Normality of data was tested with Kolmogorov-Smirnoff & Lilliefors test. Because all variables obtained were out of normality, most used tests were non parametric. However, to increase the statistical information when it was possible all variables were transformed (root squared or log) in order to run ANOVA main effects test. Non parametric tests (Friedman, Kruskal Wallis and Wilcoxon paired test) were used to assess heterogeneity by sampling sites (between sediment cores of one sampling station), by the entire wetland (all sampling sites) or to test the effect of time in rates. Non parametric Spearman rank R and Kendall Tau correlations were used to assess the relationship between variables. Results were considered as statistically significant for p-value < 0.05. All statistical analyses were performed with STATISTICA v.7 (StatSoft Inc., Tulsa, USA).

4. RESULTS

4.1. Environmental Settings at Sampling Sites

Water quality in the sampling sites during the summer 2012 is shown in **Table 4.1**. Mean inundation at TDNP during this sampling year was 1,450 ha (>80% of its maximum inundation), ensuring therefore the complete mixing of water in the whole ecosystem. During this wet year water was flowing throughout the wetland, however, total phosphorus contents were high in most wetland sites closed to the wetland inlet area.

These sites also displayed high chl-*a* levels indicating eutrophic/hypereutrophic symptoms according to OCDE standards. High ammonium concentrations could be found at the inlet sites as related to wastewater discharges of a nearby town (Villarrubia de los Ojos).

Finally, conductivity was higher as TDNP was flooded mainly by saline sulfate-rich surface waters flowing from the Gigüela river.

Table 4.1. Water quality in sampling sites of TDNP during summer 2012.

		Site-1	***Site-2***	***Site-3***	***Site-4***	***Site-5***	***Site-6***	***Site-7***	***Site-8***
Total solids	(mg/l)	2168	2432	2124	1800	1640	1900	1896	1580
Tot-Org. matter	(mg/l)	376	440	504	288	284	292	384	240
Tot-N	(mg N /l)	1.82	2.54	0.848	1.321	0.875	1.38	0.793	0.805
TDN	(mg N /l)	1.33	2.43	0.681	1.273	0.856	1.20	0.732	0.732
NO_3^-	(mg NO_3^- /l)	2.73	8.36	0.156	0.102	0.142	0.106	0.120	0.195
NO_2^-	(mg NO_2^- /l)	0.177	0.710	0.003	0.003	<0.0001	<0.0001	0.003	0.003
NH_4^+	(mg NH_4^+ /l)	0.312	0.105	0.060	0.013	0.024	0.015	0.012	0.031
Tot-P	(mg P /l)	0.086	0.104	0.084	0.147	0.005	0.014	0.051	0.027
PO_4^{3-}	(mg PO_4^{3-} /l)	0.046	0.021	0.037	0.025	0.015	0.015	0.012	0.015
TOC	(mg C /l)	7.17	4.89	7.85	13.45	9.76	15.3	9.42	9.24
DOC	(mg C /l)	7.17	4.76	7.73	13.37	9.03	13.64	9.10	9.21
Chl-a	(µg /l)	54.2	187.4	8.67	11.76	4.86	2.75	2.51	0.806
pH		8.04	8.05	8.21	8.13	8.12	7.32	8.10	8.46
Conductivity	(mS cm^{-1})	2.80	3.04	2.66	2.40	2.16	2.55	2.62	2.22

Total Carbon and Nitrogen contents of wetland soils displayed not significant differences with depth (Friedman ANOVA, $p > 0.05$), ranging 15-30% and 0.1-2%, respectively (**Table 4.2**) Highest %C and %N were obtained in 20-30 cm in *Site-2*. Lowest values were found in *Site-7*, which was also very different from the rest (Friedman ANOVA, $p < 0.05$).

Table 4.2. Total Carbon and Nitrogen (%) from TDNP sampling sites by soil depth horizon (0-10 cm. 10-20 cm and 20-30 cm). Data from replicate cores (mean ± standard deviation)

Sampling Site	**0-10 cm**		**10-20 cm**		**20-30 cm**	
	%C	**%N**	**%C**	**%N**	**%C**	**%N**
Site-1	14.71 ±0.57	1.35 ± 0.02	7.07 ± 0.25	0.43 ± 0.02		
Site-2	12.75 ± 0.06	0.63 ± 0.00	13.54 ± 0.05	0.62 ± 0.00	28.60 ± 0.63	1.89 ± 0.06
Site-3	14.45 ± 0.11	0.56 ± 0.01	13.98 ± 0.14	0.39 ± 0.01	13.55 ± 0.10	0.32 ±0.01
Site-4	14.46 ± 0.25	0.59 ± 0.03	13.51 ± 0.26	0.49 ± 0.02	12.97 ± 0.23	0.44 ± 0.03
Site-5	12.79 ± 0.12	0.48 ± 0.02	11.85 ± 0.10	0.43 ± 0.02		
Site-6	12.23 ± 0.19	0.55 ± 0.02	10.83 ± 0.12	0.32 ± 0.01	13.38 ± 0.31	0.63 ± 0.02
Site-7	4.55 ± 0.06	0.19 ± 0.00	2.98 ± 0.12	0.12 ± 0.00		
Site-8	14.24 ±0.17	0.60 ± 0.00				

4.2. Redox Potential during Incubations

Redox potential in replicates of sediment cores were similar (Friedman ANOVA, $p > 0.05$). Mean redox achieved during incubations was -40.2 mV (range -271.4, +147.8 mV). Throughout incubations redox potential decreased towards more negative potentials, confirming the anaerobic condition has been achieved (**Figure 4.1**). According to depth, redox potentials below -100 mV were obtained in 0-10 cm at 24 hours; while in 10-20 cm and 20-30 cm it occurred at 72 hours. More negative redox potentials were always recorded at 144 hours in 0-10 cm (**Figure 4.1**). At 144 hours, incubated slurries reached redox potentials which favoured methanogenesis (-244.0 mV). However, until 144 hours redox potentials were between the range of Fe- and sulfate-reduction processes (-47 mV; -221 mV; respectively).

Highest reduction of redox occurred in *Site-3* in all incubated cores (**Figure 4.1**). Contrarily, incubated cores at *Site 4, 6* and *7* did not recorded larger changes on redox, but progressively.

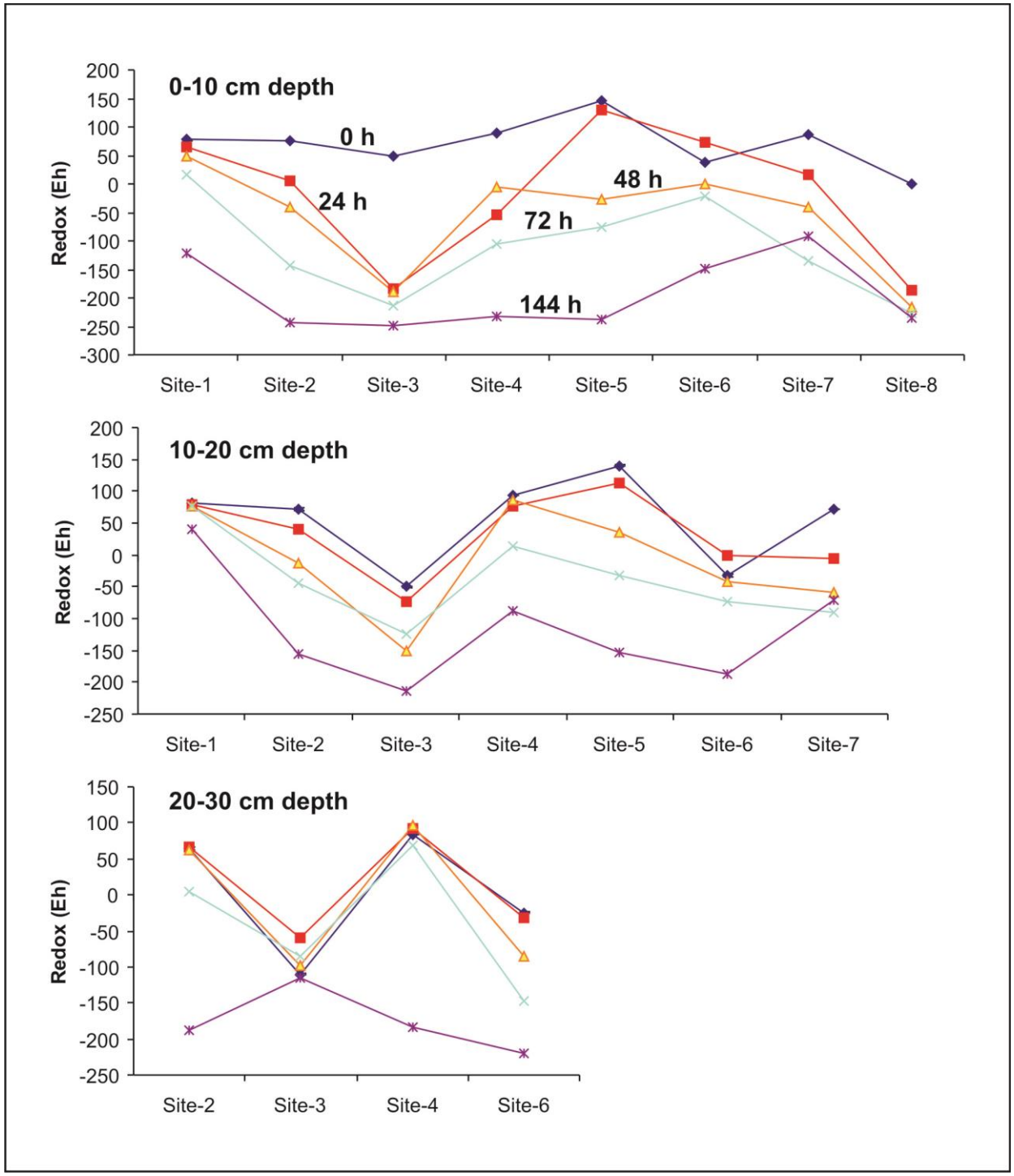


Figure 4.1. Redox (Eh in mV) during slurry incubations. Standard deviations are not represented in order to simplify the figure.

4.3. Changes on Hydrogen Sulfide, Sulfate, Ferrous and Ferric Ions during Incubations

After 144 h of incubations, hydrogen sulfide concentrations oscillated between 0-3.3 µmol/ g DW. Highest concentrations were obtained in *Site-7*, *Site-8* (0-10 cm); and *Site-1* (10-20 and 20-30 cm). Lowest ones were recorded in *Site-3* and *Site-4* (data not shown). Mean sulfide concentration changes during incubation varied between -0.005 and +0.017 µmol/ g DW at 144h, without sediment depth differences (ANOVA Main Effects, $p>0.05$). All incubated cores of 0-10 cm depth except *Site-5* experienced increases on sulfide concentrations (**Figure 4.2**). At 10-20 cm of soil depth the incubated cores *Site-5* and *7* decreased the sulfide concentrations (**Figure 4.2**). *Site-8*, *Site-6* and *Site-3* had highest values at 144 hours, in 0-10, 10-20, 20-30 cm, respectively. Lowest value was found in *Site-5* (ANOVA Main Effects, $F_{2,30}= 2.4$; $p<0.005$; **Figure 4.2**). Sulfide changes at *Site-1*, *Site-2*, *Site-6* and *Site-7* demonstrated not significant statistically changes according to time incubations (Friedman ANOVA test, $p>0.05$).

Sulfate concentrations ranged 1.6-20.6 mmol / g DW. Sulfate concentration were higher in *Site-1* and lowest in *Site-8* incubations (data not shown), as related with sulfate-rich waters of Gigüela river entering into the wetland system. Both sites exhibited significant differences in sulfate concentrations (ANOVA Main Effects, $F_{3,45} = 3.32$; $p<0.001$).

Site-1 accounted for more oxidized compounds probably due to the effect of the water inflows. At 144 h of incubation sulfate changed in the range of -4.5 to +4.3 mmol / g DW, being this tendency greater in 10-20 and 20-30 cm in *Site-4* (**Figure 4.3**), although being depth differences not significant statistically (ANOVA Main Effects, $p>0.05$).

Using the entire dataset sulfate and hydrogen sulfide concentrations were negatively correlated although weakly (Spearman Rank Order Correlation: $r= -0.46$, $p<0.05$). *Site-5* and *Site-6* exhibited sulfate consumption compared to the other sites (Kruskal-Wallis Test; $p = 0.003$). The increase of sulfide concentrations observed at *Site-6* was correlated significantly with the sulfate decrease (Spearman Rank of Correlations: $r= -0.89$, $p<0.05$). Sulfate was consumed in 39% of incubated slurries at a mean rate of 11.2 ± 12.3 µmol/g DW/ h. However, sulfate also increased in 61% of incubations at an average rate of 9.3 ± 7.8 µmol/g DW/ h. Differences between sulfate production and consumption was not related with sulfate availability of wetland soils (Spearman Rank Order Correlation $p>0.05$).

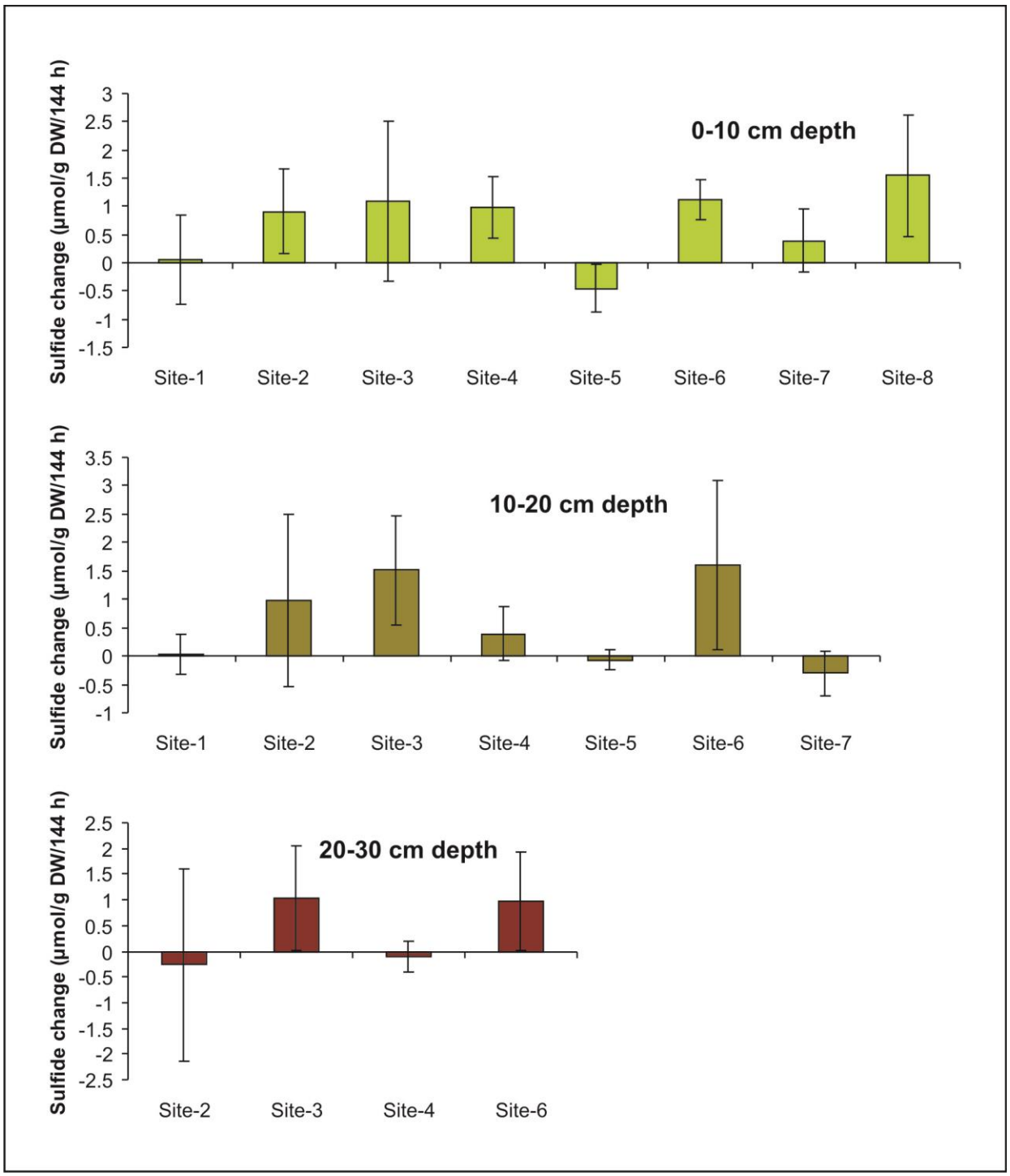


Figure 4.2. Changes in hydrogen sulfide with soil depth at sampling sites after 144 h of slurry incubations.

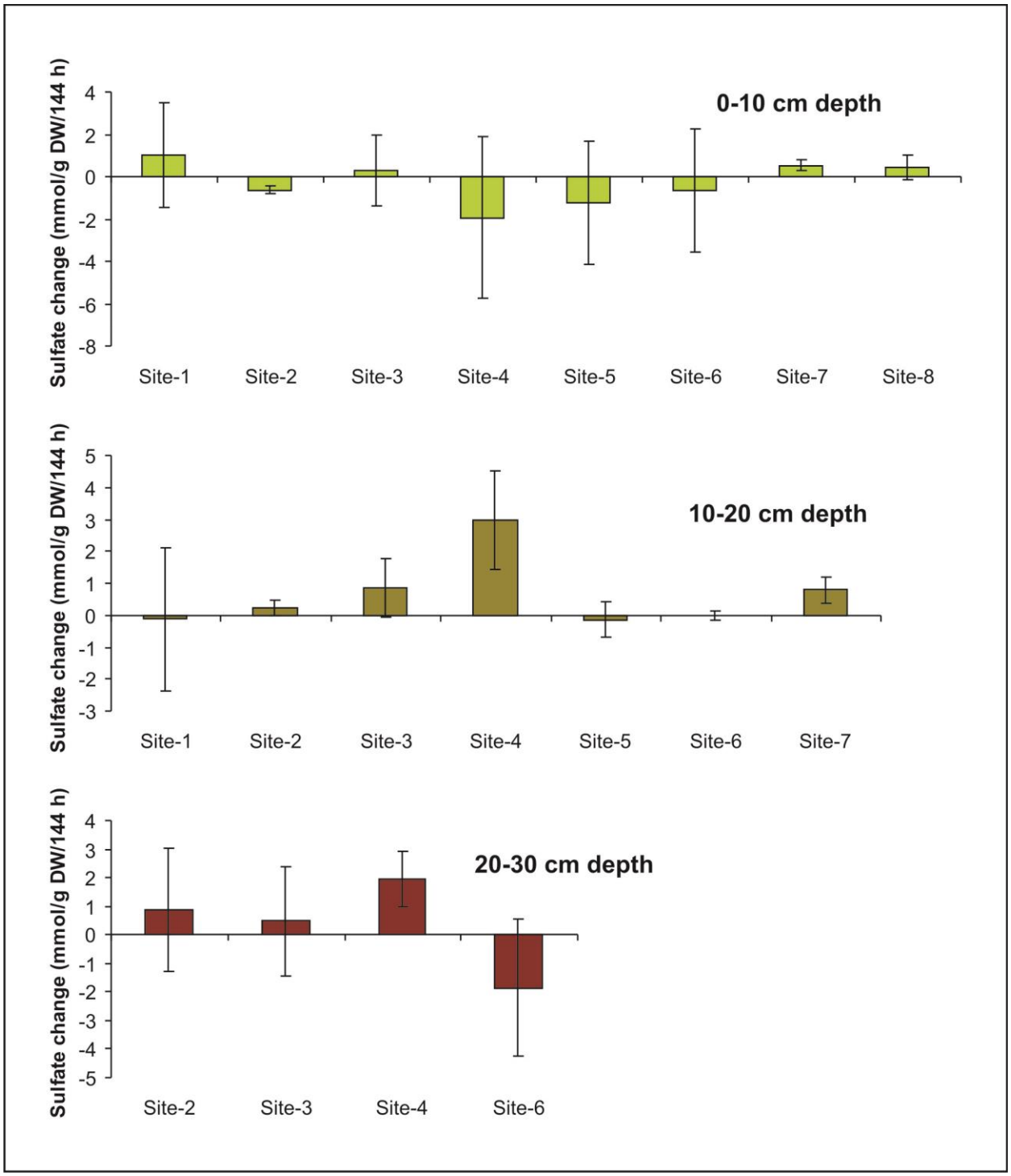
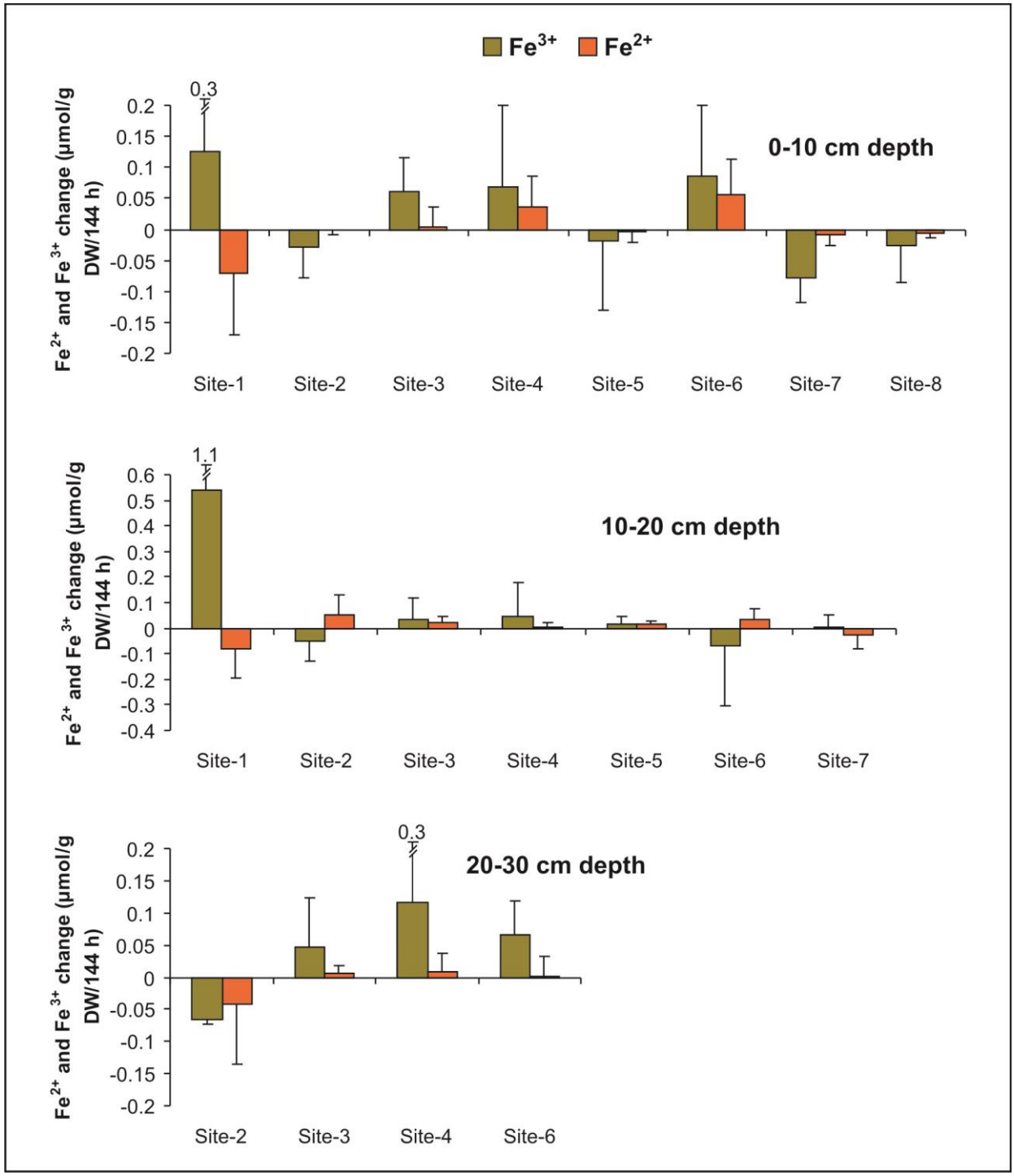


Figure 4.3. Changes in sulfate with soil depth at sampling sites after 144 h of slurry incubations.

Ferrous ion (Fe^{2+}) concentrations during incubations ranged 0-0.182 µmol/ g DW at 144 hours, with highest values registered in *Site-1*, *Site-3* and *Site-6* and lowest in *Site-4* (data not shown). Ferrous ion changes after 144 h of incubations varied between -0.155 to +0.095 µmol/ g DW, with site differences (ANOVA Main Effects, $p<0.01$; $F_{3,45} = 2.19$; **Figure 4.4**).



Figures 4.4. Changes in ferrous and ferric ions with soil depth at sampling sites after 144 h of slurry incubations.

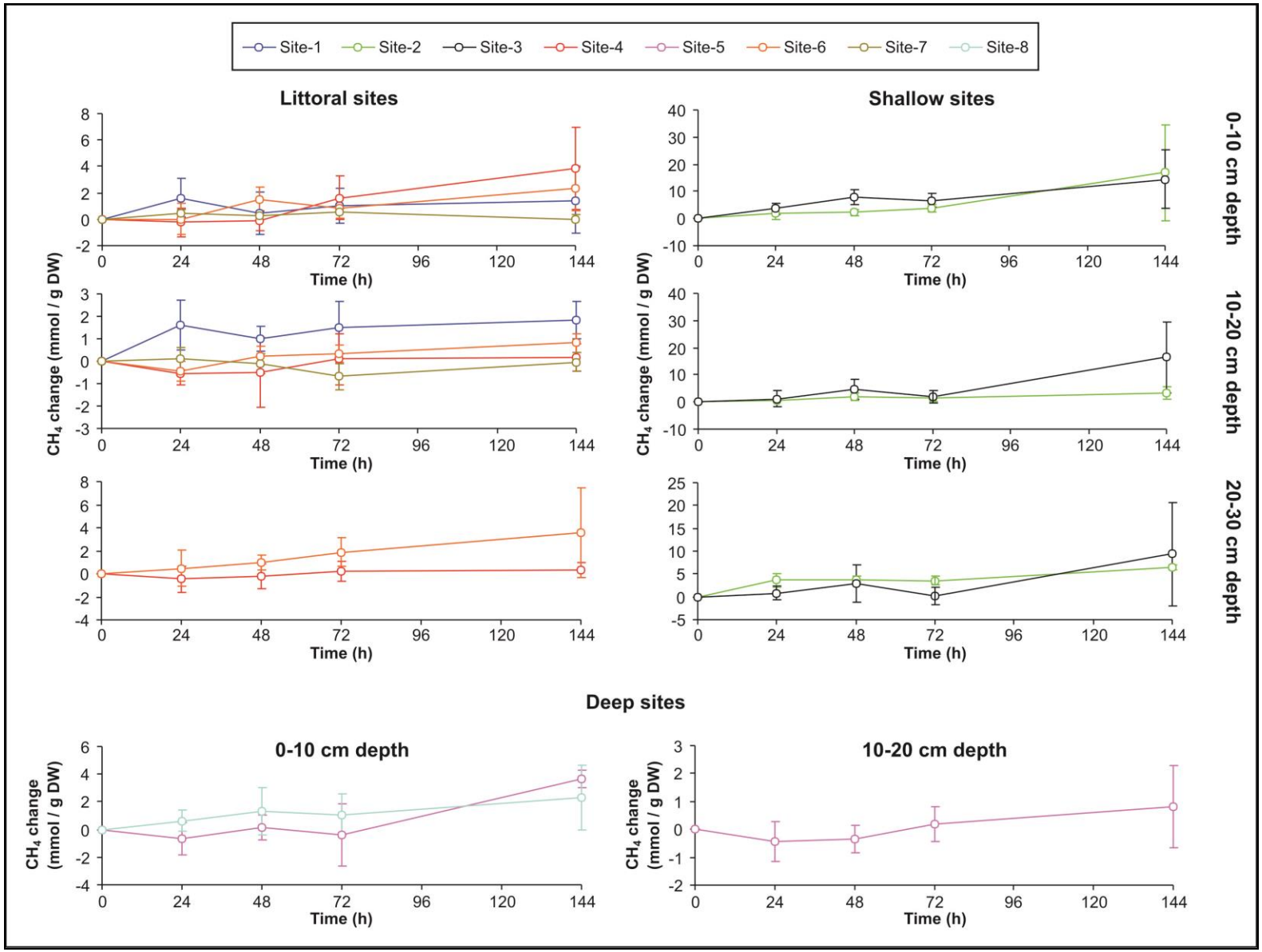
Although a decreasing on ferrous ion was found by sediment depth (Figure 4.4), these changes were not significant statistically (ANOVA Main Effects, $p>0.05$). On the other hand, ferric ion (Fe^{3+}) concentrations ranged from 0.029 to 1.110 µmol/ g DW, being higher in *Site-1* and *Site-2*. Lowest values appeared at *Site-4* at the beginning of the incubations (data not shown). Ferric ion changes varied between -0.171 to +0.950 µmol/ g DW / 144h, with significant increases at *Site-1* and *Site-4* (ANOVA Main Effects, $p<0.01$; $F_{3,45} = 2.19$; **Figure 4.4**). The reduction of ferric concentrations observed at the remaining sites resulted not significant statistically (ANOVA Main Effects, $p>0.05$).

An inverse significant relationship between Fe^{2+} and $Fe^{3+}$was found at *Site-3* incubations (Spearman Rank Order Correlations: $r= -0.84$, $p<0.05$). Using the whole dataset, both changes in ferrous or ferric ions during incubations were not significant statistically (Friedman ANOVA test, $p>0.05$). Ferrous ion demonstrated a weak significant relationship with hydrogen sulfide changes (Spearman Rank Order Correlation $r= 0.47$, $p<0.05$). Sulfate and ferrous ion were also weakly correlated (Spearman Rank Order Correlation $r= 0.33$, $p<0.05$).

4.4. Changes in Methane and Acetate during Incubations

Methane concentration in replicate cores by site demonstrated large spatial heterogeneity at fine scales on methanogenesis process (Friedman ANOVA test, $p<0.05$) except at *Site-2* and *Site-3* (Friedman ANOVA test, $p>0.05$). With soil depth, the changes in methane concentrations were not significant statistically (ANOVA Main Effects, $p>0.05$).

Methane concentration ranged, on average, from 0.001 to 0.031 µmol CH_4/ g DW after 144 h of slurry incubations. Maximum methane concentrations were obtained at 144 hours in *Site-2* and *Site-3* (0.037 and 0.055 µmol/ g DW, respectively), although some slurry incubations exhibited the highest increases to shorter period (*Site-1* and *Site-2*, at 48 hours). All slurries displayed significant changes on methane concentration during incubations (Friedman ANOVA test, $p<0.05$), except *Site-1*, *Site-4* and *Site-5*. Highest increases in methane concentrations were recorded in sites located close to the river inlet (*Site-2, Site-3 and Site-4;* **Figure 4.5**) whereas lowest values appeared at the end of the wetland (*Site-5, Site-6, Site-7 and Site-8* (**Figure 4.5**; ANOVA Main Effects, $F_{5,75} = 1.74$; $p<0.01$). On average, rates of methane production were higher at 24 and 144 hours. Methane production rates ranged from 0 – 0.22 nmol CH_4 /g DW/ h, with the highest rates found in *Site-3*. (ANOVA Main Effects, $F_{4,60} = 2,08$; $p<0.01$).



Figures 4.5. Changes in methane concentration with soil depth at sampling sites during slurry incubations.

Negative values on methane production rates were shown between -0.056 to -0.001 nmol CH_4/g DW/ h, with an irregular temporal trend during incubations. Decline on methane concentrations were correlated with increases on redox *Eh* (Spearman Rank Order Correlation: r=-0.42, p<0.05) in *Site-3* (20-30 cm), *Site-4*, *Site-5* (0-10 cm), and *Site-6*, *Site-7* (10-20 cm). Negative changes on methane concentration accounted up to 25% of the methane production.

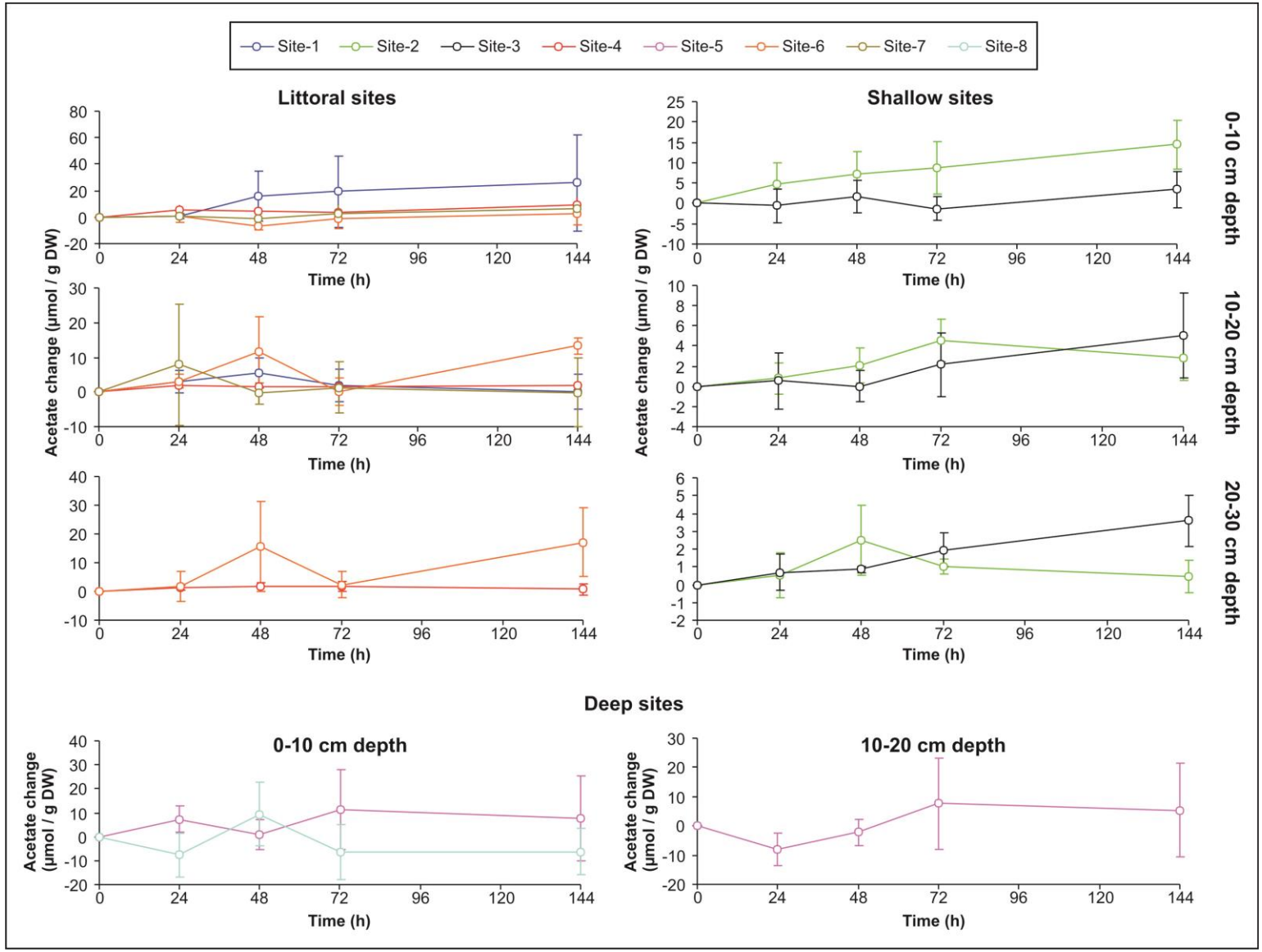
Acetate concentrations were in a range between 4.6 to 88.2 µmol / g DW. After 144h of slurry incubations rather than being actively consumed during microbial processes, acetate increased at an average rate oscillating 3.5 (10-20 cm) and 5.5 (0-10 and 20-30 cm) µmol/ g DW. Changes on acetate concentration ranged from -13.5 to +55.0 µmol / g DW (**Figure 4.6**). Highest increases were recorded in *Site-1* and *Site-2* at 0-10 cm of soil depth, in *Site-5* at 10-20 cm and in *Site-6* at 20-30 cm, throughout a gradient from NE to SW of the wetland system. Site differences in changes on acetate concentration were found (ANOVA Main Effects, $F_{4,60} = 0.001$; $p<0.05$)

Inverse weak relationships were statistically significant between methane and acetate concentrations at 24, 48 and 144 h (Spearman Rank Order Correlations: r= -0.22, r= -0.27 and r= -0.28, respectively, p<0.05). However, also positive relationships were asserted as in *Site-1* and *Site-2* (Spearman Rank Order Correlations: r= 0.67 and r= 0.72, respectively, p<0.05).

Methane production/consumption depended although weakly on soil carbon and nitrogen contents in all incubation periods (**Table 4.3**). Acetate changes did not depend on carbon and nitrogen contents (**Table 4.3**).

Table 4.3. Relationships (Spearman Rank Order Correlations; r values) between rates of CH_4 production/consumption, changes on acetate concentration, carbon and nitrogen soil contents (%).Marked correlations are significant at p-level < 0.05.

	%C	%N
Rates of CH_4		
24 h	**0.420**	**0.374**
48 h	**0.465**	**0.412**
72 h	**0.537**	**0.550**
144 h	**0.490**	**0.488**
ΔC of Acetate		
24 h	0.159	0.032
48 h	0.000	-0.032
72 h	0.177	0.209
144 h	0.158	0.077



Figures 4.6. Changes in acetate concentration with soil depth at sampling sites during slurry incubations.

Sulfate reduction process resulted more feasible under the anaerobic conditions obtained during slurry incubations (**Table 4.4**). Using the entire dataset changes between sulfate and methane concentrations were negatively correlated although the relationship was not significant statistically (**Table 4.4**). Only *Site-2* displayed a stronger and inverse relationship between methane and sulfate changes (Spearman Rank Order Correlations: r= -0.89, $p<0.05$).

Contrarily, sulfate and acetate were strongly correlated (**Table 4.4**). A significant correlation appeared between changes in Fe^{2+} and hydrogen sulfide (**Table 4.4**). Changes in Fe^{3+} did not show any significant correlation with H_2S after 144 h of incubations, although some sampling sites exhibited individually significant relationships but of distinctive sign (*Site-4* vs. *Site-5*, Spearman Rank Order Correlations: r= -0.89 and +0.89, respectively).

Curiously, *Site-4* and *Site 5* also displayed significant correlations between Fe^{3+} and acetate changes (Spearman Rank Order Correlations r = 0.83 and -0.94). Finally, correlations between CH_4 and Fe^{3+} were only positively correlated at 72 hours while higher negatively correlation of acetate and ferric ion was achieved at 72 hours and 144 hours.

Table 4.4. Relationships (Spearman Rank Correlations; r values) between sulfate and hydrogen sulfide concentrations and CH_4, acetate, H_2S, Fe^{2+} and Fe^{3+} concentrations at 144 hours. (Marked correlations are significant at $p < 0.05$).

	Sulfate	**H_2S**
CH_4	-0.088	--
Acetate	**0.617**	--
H_2S	**-0.463**	--
Fe^{2+}	**0.333**	**0.473**
Fe^{3+}	-0.020	-0.04

5. DISCUSSION

5.1. Methane dynamics and biogeochemical relationships

Water quality of TDNP during the study period was eutrophic/hypertrophic in sites located in the upper portion of the wetland according to OECD standards. The ecosystem processes appears clearly biased by the wetland areas impacted by masive nutrients inputs (i.e. inflows of poorly or null treated wastewater) located close to the Gigüela river inlet. Despite this spatial heterogeneity, soil carbon and nitrogen contents were homogenously distributed throughout the wetland except in one site (*Site-7*), which was completely dry during the last 6 years before 2011. This picture impinges on the activity of studied biogeochemical processes which increases its degree of variability in nutrient-rich sites compared to unimpacted ones. Heterogeneity in many wetland ecosystems enable the reduction processes to occur simultaneously (Keller and Bridgham, 2007) with many factors that controls anaerobic metabolisms interacting also at a time (water table position, temperature, pH, soil carbon quality and nutrients; Segers, 1998).

Few studies on methane dynamics in freshwater wetlands have been undertaken and less on those located in semi-arid regions. Segers (1998) in their review paper cited that methane production rates in wetlands vary from 0.01 to 10 µmol/ m^3 /s. Our results are out of this range as maximum rate of methane production was close to 50 µmol/ m^3 /s (mean soil bulk density 0.8 g/cm^3, Sánchez-Carrillo et al., 2000). Clearly our measured rates using incubations were lower than those of other peatlands and swamps but in the range of some temperate lakes (**Table 5.1**).

The decline in methane production rates with depth has been cited in numerous studies (Yavitt et al., 1988, 1990; Sundh et al., 1994; Moore and Dalva, 1997; Megonigal and Schlesinger, 2002). The lack of significant differences in methane production rates with depth in our incubations might indicate the high potentiality of our wetland soils to methanogenesis although probably a major fraction of this methane could be consumed before it reaches the surface soils by reverse methanogenesis. In fact, methane production depends on soil carbon content and the carbon and nitrogen content of our soils did not vary statistically with depth.

In a general basis, short-time experiments ensure that depletion of an inhibition electron acceptor did not occur during the incubation (Dunfield et al., 1993; Nedwell and Watson, 1995). Our results confirm this observations as an increase of methane production rates was recorded throughout incubation experiments. Decline in methanogenesis was always related to increases on redox by oxygen contamination of vials. However, methanogenesis under elevated redox (lower negative *Eh* values) can be found in the literature (Bachoon and Jones, 1992; Kludze and DeLaune, 1994).

Table 5.1. Methane production rates in some wetlands.

Site	Latitude (°N)	CH_4 (nmol/g/h)	Temp (°C)	Depth (cm)	Reference
Tablas de Daimiel (Central Spain)	39	0-0.22	25	0-30	This study (2013)
Pocosin-swamps (North California, USA)	35	0.19-0.5	25	0-20	Bridgham and Richardson (1992)
Peatlands (Upper Peninsula of Michigan, USA)	46	1.6-98.8	11-17.5	0.30	Keller and Brigham (2007)
Lakes Mårn and Lillsjön (Sweden)	65	0.1-15	20	0-5	Bastviken et al., (2003)
Lakes Ljustjärn, Lilla Sångenand Svarttjärn (Sweden)	65	0.083-166	0-10-20-30	-	Nguyen et al. (2010)
Lake Constance (Germany/Switzerland, Austria)	47	0.15-0.20	20	0-10	Rothfuss et al., (1997)
Lake Biwa (Japan)	34	0.20-0.39	6.8	0-15	Dan et al., (2004)

Methane production rates and C:N ratios were related statistically as also observed by. Nguyen et al. (2010) in Swedish lakes. The relationship between methanogenesis and soil nitrogen contents indicates that a large labile carbon pool is available in the soil which is consumed preferentially by nonmethanogenic bacteria until the inorganic electron acceptor pools are exhausted and therefore favouring methanogenesis at the end (Megonigal et al., 2004).

Slurry incubations were always promoting the anaerobic formation and accumulation of acetate. Acetate accumulation was around 1000-fold the methane production rates. Large acetate accumulations were previously found by Hines et al. (2001), Duddleston et al., (2002) and Keller and Brigham (2007) in peatlands, where acetate accumulated from 200 to 800 µM after 8 days of incubations under anaerobic conditions. In our study, acetate accumulation during incubations was slightly lower ranging 50-500 µM after 6 days. This indicated that changes on methanogenesis were not related with substrate limitation as acetate was always in excess. The accumulation of potential methanogenic precursors might indicate a decoupling of terminal metabolism from fermentation and other reactions that funnel carbon to terminal steps (Hines et al., 2008). Therefore, acetate may be the terminal step, rather than methanogenesis. Both the weak inverse relationships between methane and acetate concentrations and the positive correlation in some nutrient-rich sites could be confirming this hypothesis. Duddleston et al. (2002) found that the use of specific substrates or inhibitors may inhibit acetoclastic methanogens relative to H_2 utilizers, increasing the acetate accumulation after anaerobic incubations. However, these compounds were not used in our incubations. Acetate could have been originated from fermentation, acetogenesis, or sulfate reduction processes (Odom and Singleton, 1993; Duddleston et al., 2002).

Sulfate reduction process resulted more feasible than methanogenesis under anaerobic conditions obtained during slurry incubations. Although not significant statistically, sulfate reduction occurred as opposed to methanogenesis (*Site-2* displayed a stronger and inverse relationship), acetate and sulfate concentrations were strongly correlated. The strong relationship between sulfate and acetate suggest that the capability of methanogens competing with other acetogenic bacteria such as sulfate-reducers was weaker in this wetland site.

The importance of sulfate reduction for wetland biogeochemistry has remained underestimated because standing pools of sulfate are typically in the lower µM-range to sustain sulfate reduction over longer periods of time (Dedysh et al., 2006). In some wetlands sulfate reduction may operate at rates that are comparable to marine surface sediments (Jørgensen, 1982; Smemo and Yavitt, 2010; Pester et al., 2012). Barlett and Harris (1993) reported that only in the range of 10 to 30 µM SO_4^{2-} methanogenesis became the dominate pathway for carbon decomposition. In moderately sulfate-rich soils as in Florida Everglades (> 200 µM) sulfate reduction rates were 0.10 µmol/g DW/ h (D'Angelo and Reddy, 1999). In TDNP wetland the SO_4^{2-} soil content is very high (2-21 mM) and the mean rate of sulfate reduction was 100-fold higher (11.2 ± 12.3 µmol/g DW/ h; data from incubated cores with decline in sulfate concentrations).

Sulfate reduction process in TDNP did not depend on sulfate availability of soils. Sulfur cycling in wetlands could occur from a constant external input of sulfate (e.g.: groundwater or surface inflows, atmospheric deposition) or by a recycling mechanism of reduced compounds to sulfate (Pester et al., 2012), which appear coupled or decoupled to short-term (Knorr and Blodau, 2009). The second pathway has been suggested to be the main driving force on sulfate cycling in many wetlands (Blodau et al., 2007), being related to the water-level changes (i.e. changes on redox providing electron acceptors; Fleckenstein et al., 2011). The spatial difference observed on changes in sulfide concentrations (inverse to methanogenesis; high values in deep zones at the wetland end sites) confirms the importance of water level as the key control on sulfate reduction as observed in other wetland sites (Mandernack et al., 2000; Alexwell et al., 2006).

From a thermodynamic point of view, the degradation of organic matter in sulfate-reducing environments is different from the degradation in methanogenic environments. In contrast to sulfate reducers, methanogens use a limited number of substrates for growth. Quantitatively, hydrogen, carbon dioxide and acetate are the most important and best-known susbtrates for methanogens while lactate, propionate and butyrate are for sulfate-reducers. However, in the presence of an excess of sulfate as in our wetland, methanogens compete with sulfate reducer bacteria for the common substrates: hydrogen and acetate (Muyzer and Stams, 2008). Sulfate reduction may be diverting anaerobic carbon mineralization away from methanogenesis.

It is not clear whether negative methane production rates registered in our incubations respond to reverse methanogenesis or to insufficient oxygen-free purging during slurry preparations. The lack of significance in the relationships between both sulfate and methane or sulfide and methane concentrations might dismiss this hypothesis as anaerobic methane oxidation is coupled to sulfate reduction (Knittel and Boetius, 2009; Smemo and Yavitt, 2011).

Fe^{2+} can account for up to the 90% in wetland anoxic sediments (Roden and Wetzel, 1996, 2002). In peatlands, high values of iron ions have been cited (10mM Fe^{2+} and 0.5mM Fe^{3+}) compared with bogs sites (20µM Fe^{2+} and 5µM Fe^{3+}; Smemo and Yavitt, 2011). In TDNP concentrations of iron ions were extremely lower (0.21 µM Fe^{2+} and 0.85 µM Fe^{3+}) which could be impacting on the ferric reduction process. Moreover, a strong interaction between the anaerobic S cycle and the Fe cycle has been found in some saltmarsh wetlands (Kostka et al., 2002), which might regulated the sulfur availability for sulfate reducer bacteria.

Our results indicate that Fe^{2+} concentration in TDNP soils after incubations (0.04 µmol Fe^{2+}/g DW /144 h) are lower than those recorded in short-time incubations with soils from the Florida Everglades (0.4 ± 0.0 µmol Fe^{2+} / g DW, after a methanogenic conditioning; D'Angelo and Reddy, 1999). These authors found that the elevated high Fe^{2+} content is caused by chemical reduction with accumulated H_2S. Reduced products of both processes (Fe^{2+} and H_2S) usually form ferrous sulfides (Mitsch and Gosselink, 2007). The weak relationship exhibited between Fe^{2+}:H_2S (r = 0.38) in our experiments might explain the lower Fe^{3+} reduction rates occurring in TDNP. As a matter of fact, ferric ion displayed one order of magnitude greater than ferrous ion.

The availability of Fe^{3+} has been cited as a factor controlling ferric reduction (Weiss et al., 2004). In fact, Fe^{3+} accumulates mainly close to the rhizosphere in saltmarsh soils (500 µmol Fe^{3+}/g DW in the rizhosphere versus 50 µmol Fe^{3+}/g DW in no-rooted soils) as related to oxygen transport by wetland plants to the soil (Weiss et al., 2004). Clearly, this distinction was not accounted in our study but the littoral samples demonstrated not different content on Fe^{2+} and Fe^{3+} which must have contained high plant roots.

5.2. Biogeochemical Budget

In **Figure 5.1** represents the biogeochemical budgets from the main pathways analysed.

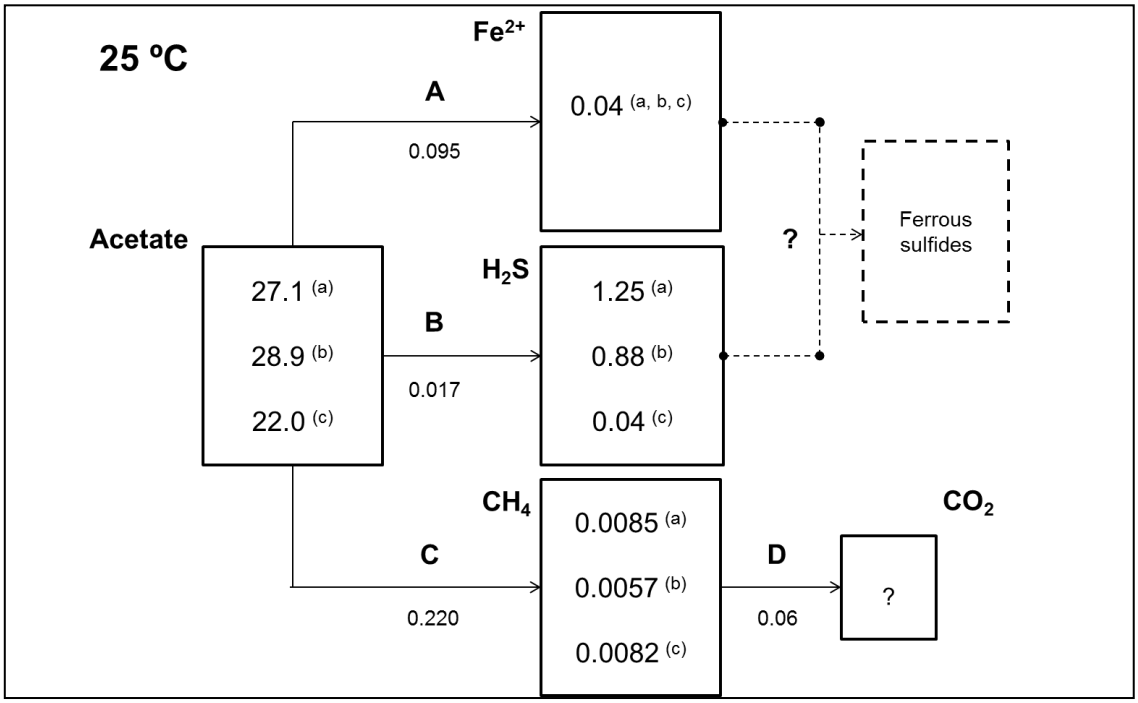


Figure 5.1. Biogeochemical budget proposed between the initial concentrations of acetate (i.e.: substrate) and final concentrations of reduced compounds obtained in incubated sediment slurries from TDNP under anaerobic conditions at 25ºC. The concentrations of reduced compounds (rectangles) are expressed in µmol / g DW after 144 h of incubations. Maximum flow rates are expressed in µmol / g DW /144 h (except for methane rates, nmol / g DW / h) for each reaction (A-D): A) Ferric reduction, B) Sulfate reduction, C) Methanogenesis, D) Methane oxidation or reverse methanogenesis. Letters in brackets correspond to sediment depth horizons (a, 0-10cm; b, 10-20cm; c, 20-30 cm).

It has been suggested that at 25ºC the main substrate compound analysed, acetate, was split into the following main reactions (in arrows): A) Ferric reduction, B) Sulfate reduction, C) Methanogenesis; and indirectly D) Methane oxidation or reverse methanogenesis. Hence a minor fraction of acetate was consumed by acetoclastic methanogens. This biogeochemical budget does not account for the on-going production of fermentation products, neither in a stoichiometric sense nor in thermodynamic calculations, it only accounts to concentrations and rates (or changes in concentrations) previously discussed. The figure aims to summarize the main biogeochemical processes described in this study, and lacking from others (i.e.: H_2-reduction methanogenesis). Following Metje and Frenzel (2005), other complex organic matter and/or intermediates could play a major role (i.e.: ethanol).

On an ecosystem scale, freshwater wetlands are recognised as a major source of the GHGs. **Figure 5.2** represents only methane dynamics as a half of the methane process, as net methane emissions must be calculated from both production and consumption. Therefore, annual emission rates must be considered as potential ones while reverse methanogenesis and methane transport from soils to the atmosphere will be assessed.

Using the spatial pattern emerging on methane and sulfate reduction process in Las Tablas, calculations were accounted dividing the entire wetland into two main zones: Las Tablas zone (*Site-1, Site-2, Site-3, Site-4*) and Las Cañas zone (*Site-5, Site-6, Site-7, Site-8*). Moreover, this division includes the main degradation/restoration plans developed in the wetland from 1940s as well as the effects of the hydrological and water quality changes during the last 50 years (Sánchez-Carrillo and Angeler, 2010).

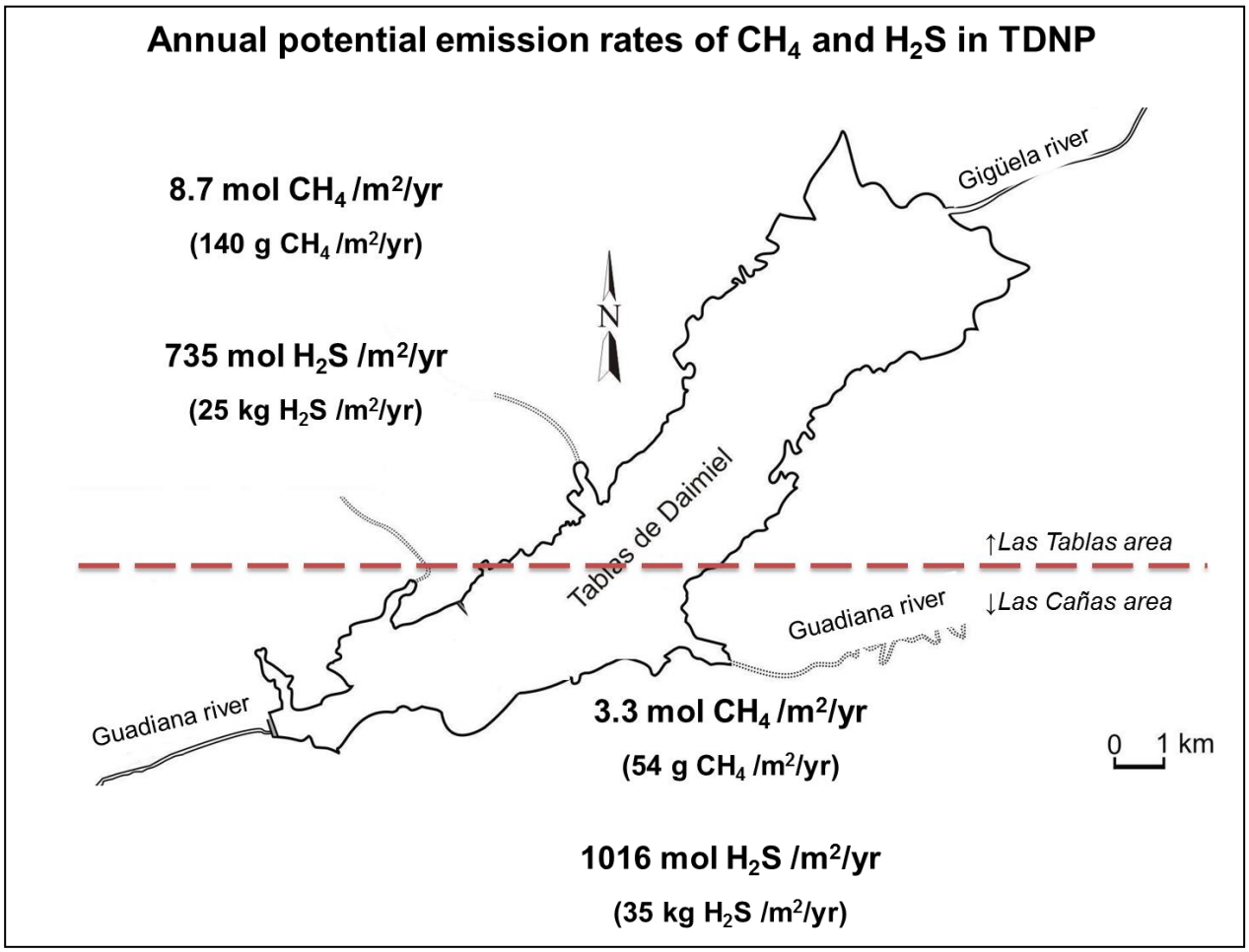


Figure 5.2. Annual potential emission rates of CH_4 and H_2S in TDNP. Conditions based on: a typical wet year (2012), potential redox (Eh) of -200 mV and the upper sediment horizon (0-10 cm).

On average, mean methane emission by wetlands is in the range of 0.07-19,044 g $CH_4/m^2/yr$ (Ortiz-Llorente and Álvarez-Cobelas, 2012). Our potential methanogenesis rates were in the lower range of methane emissions reported in that review study (54-140 g $CH_4/m^2/yr$).

Usually freshwater wetlands are considered as lower potential producers of hydrogen sulfide except those highly reduced, unvegetated, sulfide-rich wetlands such as brackish marshes (Megonigal et al., 2004). No data is available on annual estimates of sulfide emission rates by wetlands and our results could not be compared. Anyway, it is to expect that the large sulfate concentration on TDNP soils probably positioned this wetland in the higher range of sulfide emissions of freshwater ecosystems.

The relationship between methanogenesis and sulfate reduction processes in wetlands like TDNP demonstrate the importance of the anaerobic S-cycle suppressing the methane emissions as observed in other wetlands subjected to acid deposition (Gauci et al., 2004).

6. LIMITATIONS OF ANALYSES

Biogeochemical process rates are often decoupled from one to another, making it inherently difficult to predict efflux rates from environmental studies. This is due to the interaction between production, consumption, storage and transport. Therefore, experimental controlled studies allow to study individual processes avoiding these complex interactions. However, extrapolations of these results to processes at ecosystem scales must be done carefully. Our results indicate the potentiality of the studied biogeochemical processes but rates must be taken considering the experimental conditions in which were conducted (Segers, 1998; Le Mer and Roger, 2001).

Incubation techniques did not reproduce the natural *in situ* conditions and estimated rates must be considered as influenced by the coring technique and the incubation conditions such as shaking and exclusion of atmospheric oxygen input (Kelley et al., 1995).

7. FUTURE PROSPECTS

Future research should be conducted to incorporate some lost processes (reverse methanogenesis, H_2/CO_2 reductions, changes with oxygen supply, etc.) in order to complete the interaction between production, consumption, storage and transport of carbon in this wetland soils. The use of specific inhibitors (e.g., BES as methanogenesis inhibitor) will improve our knowledge of the methane dynamics (van der Nat et al., 1997, 2000). Furthermore, the use of intact cores will improve the results as the processes occur in undisturbed soils, allowing a more feasible extrapolation to the ecosystem scale.

Long-term measurements of methane emissions using isotopic tracers ($^{13}C/^{12}C$) could also improve our understanding on the methane (and total carbon) cycle in this wetland. The effects of global warming on methane and sulfide emissions by wetlands also need to be explored through laboratory tests. The relationship between methanogenesis and sulfate-reduction under new environmental scenarios must be revisited in future researches in order to develop mechanistic approaches which could be used in ecosystem modelling.

Finally, the role of wetlands in the CH_4 emissions have to be assessed in relation with different environmental settings as these ecosystems can be used as sentinels of the global change.

8. CONCLUSIONS

- The study conducted in the semiarid wetland Las Tablas de Daimiel National Park (TDNP) has provided valuable insights into how biogeochemical processes are operating under anaerobic conditions, and especially, our results has remarkably concluded the low methane production rates.

- Environmental settings has evidenced that TDNP has two main zones: nutrient-enriched (*Sites 1-2-3-4*) and poor-enriched ones (*Site 5-6-7-8*) that were a matter of great concern which explained the heterogeneity in the biogeochemical processes.

- A major fraction of the methane production could be consumed before it reaches the surface soils, by reverse methanogenesis as depth differences were not significant statistically.

- Methanogenesis had a dual time-pattern, according to short-term (0-48 h) and long-term (0-144 h) sediment slurry incubations, indicating the relationships with other anaerobic processes could be supressing or inhibiting methane formation.

- On an ecosystem scale our potential methane emission rates were lower than other found in the literature.

- Instead of being effectively consumed by methanogenesis, slurry incubations analyses shown the formation and accumulation of acetate 1000-fold the methane production rates.

- Acetate was a non-limiting substrate to methanogenesis, therefore, acetate may be the terminal step, rather than methanogenesis.

- Ferric reduction process was lower than those recorded in short-time incubations in other wetlands. The weak relationship exhibited between Fe^{2+}:H_2S in our experiments might explain the lower Fe^{3+} reduction rates occurring in TDNP. As a matter of fact, ferric ion displayed one order of magnitude greater than ferrous ion.

- Sulfate reduction might be diverting anaerobic C mineralization away from methanogenesis as it was effectively reduced in hydrogen sulfides. The spatial difference observed on changes in sulfide concentrations confirms the importance of water level as the key control on sulfate reduction.

- Sulfate reduction was the main anaerobic process in TDNP (sulfates were in the mM-range). TDNP soils probably positioned this wetland in the higher range of sulfide emissions, being better compared with salt marshlands than freshwater wetlands.

Future research should be conducted to incorporate some lost biogeochemical processes in this wetland soils. The relationship between methanogenesis and sulfate-reduction under new environmental scenarios must be revisited in order to develop mechanistic approaches helping ecosystem modelling. Finally, the role of wetlands in the CH_4 emissions have to be assessed in relation with different environmental settings as these ecosystems can be used as sentinels of the global change.

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A. APPENDIX

Table 1. Summary of concentrations of methane, acetate, ferric and ferrous ion, sulfate and hydrogen sulfide during slurry incubations at 144 hours.

Site	Depth	CH_4	Acetate	Fe^{3+}	Fe^{2+}	SO_4^{2-}	H_2S
	(cm)			(µmol / g DW / 144 h)			
Site-1	0-10	0.005	53.25	0.423	0.022	16.80	0.690
	10-20	0.004	43.27	0.809	0.042	14.05	0.159
Site-2	0-10	0.020	21.09	0.099	0.026	6.34	1.358
	10-20	0.005	8.74	0.096	0.070	5.88	1.974
	20-30	0.008	8.74	0.168	0.065	9.16	2.211
Site-3	0-10	0.017	14.96	0.120	0.017	7.00	1.131
	10-20	0.019	20.64	0.109	0.028	6.41	1.512
	20-30	0.015	19.28	0.048	0.010	5.60	1.038
Site-4	0-10	0.008	38.20	0.106	0.038	10.31	1.023
	10-20	0.004	41.18	0.115	0.011	14.36	0.399
	20-30	0.003	38.09	0.156	0.029	14.03	0.065
Site-5	0-10	0.007	54.47	0.090	0.022	10.01	0.348
	10-20	0.004	46.13	0.080	0.019	8.50	0.102
Site-6	0-10	0.006	30.79	0.243	0.142	9.79	2.074
	10-20	0.002	25.17	0.109	0.059	4.46	1.925
	20-30	0.006	40.65	0.181	0.081	8.06	1.590
Site-7	0-10	0.002	35.95	0.036	0.042	8.08	1.349
	10-20	0.002	47.33	0.162	0.016	9.10	0.084
Site-8	0-10	0.004	33.08	0.054	0.014	4.29	2.026