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First evaluation of the marine invasive species *Spartina anglica* as a potential renewable source of glycine betaine

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ABSTRACT

The invasive species, *Spartina anglica* C. E. Hubbard from Arcachon lagoon (common name English cordgrass) was evaluated for its glycine betaine (GB) content and compared with the native *S. maritima*. Seven collections were made over a 29-month period starting from January 2009 until May 2011. Aqueous methanolic extracts were prepared from both leave and rhizome tissue of the two species. Quantitative determination of the GB concentration in the crude extract was performed by ^{1}H NMR, which offers a rapid and efficient way for precise determination of the amount of natural products in a single analytical step. The highest concentrations of GB were systematically found in *S. anglica* leaves, for which the GB content varied in the range 21.94-27.61 mg (gdw)$^{-1}$ without apparent significant seasonal effects. The rhizome contents were notably lower 3.41-12.69 mg (gdw)$^{-1}$, but remained of interest. Compared to sugar beet, which is the traditional resource of GB, the content of *S. anglica* was found up to 3 times greater. These values make *S. anglica* a raw material of interest for agricultural, dietary and pharmaceutical applications. The fact that *S. anglica* biomass may provide some economic benefits could help for developing future sustainable management plans of this worldwide invasive species.

INTRODUCTION

Spartina Schreb. (subfamily Chloridoideae of Poaceae) is a relatively small genus consisting of approximately 15-17 species, most of which (13) are native to the New World. During the19th century, various members of the genus have spread outside of their native boundaries and become invasive in their new settlement. This is especially the case of *S. alterniflora* and its derivatives, *S. anglica* and *S. townsendii*. *S. anglica* C. E. Hubbard (English cordgrass) is an extremely vigorous species, which arose in England at the end of the 19th century by a cross between the native European species *S. maritima* and the introduced eastern North American *S. alterniflora* Loisel. After the resulting sterile hybrid *S. townsendii* doubled in chromosome number, the species became able to sexually reproduce as a new species (Baumel *et al.*, 2002; Nehring and Hesse, 2008).

At first, *S. anglica* was considered a useful plant because of its ability to trap large amounts of sediment, its rapid clonal growth, strong competitive and reproductive capability, and high tolerance for a range of tideland habitats, including low or high salt marsh, mudflats and cobble beaches. As a result, *S. anglica* has been intentionally introduced to coastal and estuarine mudflats throughout the world to aid in foreshore protection, reclamation of land and stabilisation of marshes. Only the introductions into South America and South Africa were unsuccessful.

Some of the very traits that make *S. anglica* valued are also the greatest causes for concern. Through a combination of intentional planting, natural and accidental dispersion, it has become a notorious invasive plant throughout the world. *S. anglica* is a very aggressive species capable of establishing in different habitat types and transforming them. It is very effective at producing and dispersing seeds and resisting mechanical removal by storing a reserve of nutrients in large underground root masses. *S. anglica* sediment accretion rates are higher than those of other salt marsh vegetation and other *Spartina* species As a result, *S. anglica* causes tidelands to rise more than they would if they were unvegetated or vegetated by other species. In addition to marked intertidal elevation changes, the densely spaced stems of *Spartina* reduce the amount of light reaching the underlying sediments.

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The cumulative effect of these changes is major alteration of coastal and estuarine ecosystems that is detrimental to native species. The *S. anglica* invasion and spread lead to the exclusion of native plant species resulting in significant harmful ecological impacts.

All these negative effects have resulted in the nomination of *S. anglica* as among 100 of the "World's Worst" invaders (Invasive Species Specialist Group, 2000). *S. anglica* is a successful bioengineer now colonizing coastal lagoons, salt marshes and estuaries not only in its native Europe (Baumel *et al.*, 2002), but also in North America, including Alaska, Asia, New Zealand and Australia as an exotic species (see as example: Ayres and Strong, 2002). The current range of *S. anglica* is from 48°N to 57.5°N in Europe, from 21°N to 41°N in China and from 35°S to 46°S in Australia and New Zealand. The spread of *S. anglica* threatens the emblematic native species and economic interests of commercial fisheries and tourism industries (Dethier & Hacker, 2004; Nehring & Hesse, 2008), which led land managers striving to control the invasion.

The problem of invasive species and their control is one of the most pressing applied issues in ecology today. Some cordgrass eradication experiments have been reported around the world including physical removal, mowing, biocontrol and herbicide (see as example: Nehring & Hesse, 2008). Chemical control is in some cases effective, but controversial and complicated by tides. Herbicides are proven to be dangerous for environment and consumers. The European Water Framework Directive has already led to the withdrawal of many of them from the market. As a result, non-chemical means of control must be developed for future management.

The management of an invasive plant, to be real, must be sustainable. Periodical removal of the whole plant early in the growing season has proven to be effective for reducing the growth (see as example: Dethier & Hacker, 2004). However, it generates huge amounts of plant material, which are deposited in landfill site. In this context, it is important to evaluate the potential of *S. anglica* as a source of marketable products, especially as any control technique will need to be applied repeatedly, due to the dramatic resilience of *S. anglica*.

Answers may come from the recycling of the uprooted plant material as a new renewable resource for the production of bioactive substances. Except for *S. cynosuroides* (Mody *et al.*, 1974; Miles *et al.*, 1976, 1983), little is know about the metabolites of the genus *Spartina* growing under natural conditions. Greenhouse and laboratory experiments have shown the importance of glycine betaine (GB hereafter in the text) as osmoprotectant for the genus *Spartina* (Blundel *et al.*, 1992; Hester *et al.*, 2001; Mulholland and Otte, 2000, 2002).

GB has long been recognized as a versatile compound with great potential (see as example: Jokinen and Virtanen, 1996). This led us to investigate the GB content of *S. anglica* with a view toward exploiting the uprooted biomass resulting from the environmental management of the invaded area. The Arcachon lagoon was choosen as study site. Phytochemical investigation of European populations of *Spartina* in natural conditions had never been studied before. For comparison purpose, data were also acquired from samples of *S. maritima* (native and co-occuring at the same site). This paper deals with the quantitative determination over a 29-month period using quantitative NMR. This technique offers a rapid and efficient way for precise determination of the amount of compounds in a single analytical step.

MATERIALS AND METHODS

General

Trimethylsilylpropionic acid sodium salt-d9 (TMSP) and deuterium oxide used for QNMR experiments were purchased from Eurisotop. Standards GB and saccharose were obtained from the Sigma Chemical Co. (St. Louis, MO). Methanol and water used for extraction were from Aldrich Chemical Company (HPLC grade). Qualitative and quantitative NMR spectra were recorded on an AVANCE 400 MHz spectrometer (Bruker). Samples and TMSP were weighed on a Sartorius CP225D analytical balance (5 decimal places) of ± 10 μg.

Study site (Fig. 1)

The Bassin d'Arcachon (44°40'N, 1°10'W) is a mesotidal system located in the South-West coast of France.. The surface area of this coastal lagoon is approximately 185 km^2, with an exposed intertidal area of 65%. Tides are semi-diurnal. The Bassin d'Arcachon is home of the largest European *Zostera noltii* meadow, which supports high biodiversity and nurseries. This mesotidal lagoon is particularly vulnerable due to extensive areas of exposed mud flats (113 km^2), which are prime habitat for *S. anglica*. This species first described in Arcachon lagoon on 1980 has rapidly expanded, leading to increase sedimentation and important changes in vegetation cover, and there is considerable concern about the harmful impacts on this unique ecosystem. The progressive exclusion of the emblematic seagrass *Z. noltii* is in progress, especially in the inner part of the bay where the study took place. The spread of *S. anglica* also threatens the economic interests of commercial oyster fisheries and tourism industries (due to invasion into amenity areas). On 2002, hundreds of hectares were already colonized by this species and reached approximately 4500 ha by 2005 (SEPANSO). Eventually, *S. anglica* will take over the whole of the intertidal zone within 30-40 years, if left uncontrolled (SEPANSO). The study site was located in the inner part of the Bay, at Taussat (1.07.24 W, 44.72.98 N), which remains air-exposed for long periods at low tide (6–8 h). At this site, *S. anglica* and *S. maritima* cohabit.

Plant materials

The full plants of *S. anglica* and *S. maritima* were collected from January 2009 until May 2011. After collection, they were vigorously washed in freshwater for 1 to 2 min to remove sediment and salt (twice repeated), then air-dried at room temperature. Leaves were separated from rhizomes and analyzed separately. Dates of collection are listed in Table 1.

Fig. 1: View of the intertidal flats at Taussat showing the invasion of *S. anglica* in the *Z. noltii* meadow.

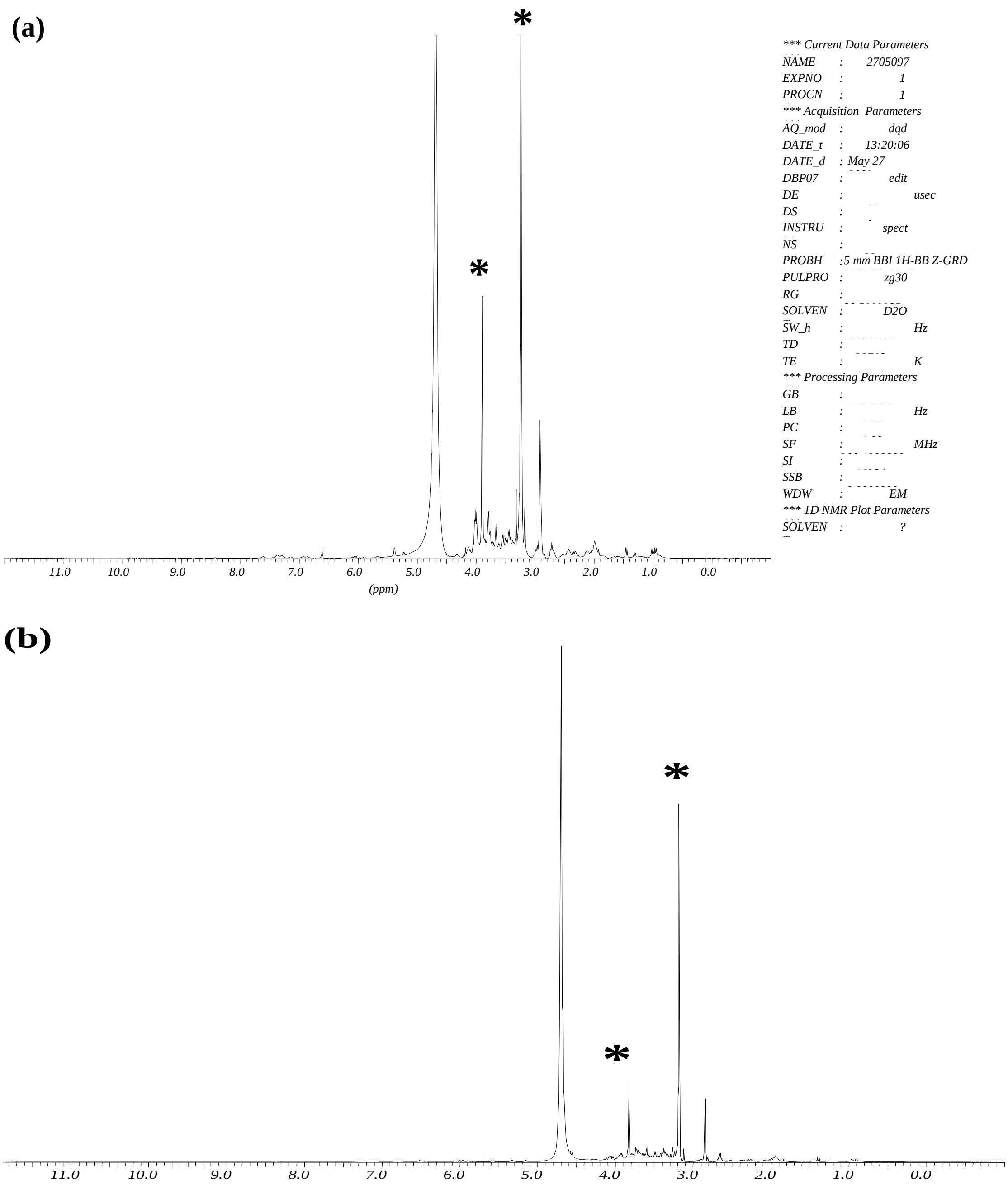

Fig. 2 : typical ^{1}H-NMR spectra (D2O) showing the GB signals(*)
(a) :Crude extract from leaves of *S. maritima* (26/04/2009)
(b): Crude extract from leaves of *S. anglica* (26/04/2009)

Extractions

The same typical procedure was applied for both leaves and rhizomes: dried finely crushed plant material (10 g) was extracted twice for 24 h in 250 mL of a 50:50 (v/v) mixture of de-ionised water and methanol at room temperature. The two resulting extracts were pooled together, and filtered. Then, the solution was centrifuged, the supernatant was evaporated *under vacuo* until complete elimination of the methanol, and the remaining aqueous solution was freeze-dried. Extraction yields are reported in Table 1.

Table. 1: Collection data and extraction yields (% $(gdw)^{-1}$).

Date of collection	*S. anglica* Leaves	*S. anglica* Rhizomes	*S. maritima* Leaves	*S. maritima* Rhizomes
27/01/09	18.3	20.5	21	20.5
26/04/09	17	21.1	16.6	21.1
28/06/10	22.5	22.7	20.6	20
22/08/10	19.5	23.5	18.7	18.8
08/11/10	20.6	25.3	19.5	26
07/03/11	-	22.8	16.9	26.9
15/05/2011	23.8	20.8	21.5	22

Determination of the GB concentrations by ^{1}H NMR

Samples were prepared with 3.00 mg of dried extract, 0.4 mL of D_2O and 100 µL of a D_2O solution of TMSP (internal standard), and transferred to a 5 mm NMR tube. The internal standard solution was prepared by dissolving TMSP of known purity in 5 mL of D_2O. One dimensional proton spectra were acquired with an AVANCE DPX 400 MHz spectrometer (Bruker). For each sample, 128 scans were recorded with the following parameters: 0.167 Hz/point; sweep width, 4401 Hz; 90° pulse width, 5.0 µs; relaxation delay, 5 s; acquisition time, 3.72 s; temperature, 300 K. Each sample was recorded in triplicate. Phase adjustments and baseline corrections were applied prior to integrations. For quantitative analysis, manual integrations of the concerned peaks were achieved. GB was quantified by integrating the area of the N-Me3 singlet at 3.27 ppm and the internal reference signal at 0.0 ppm. The amount of the internal standard was calculated to give peaks of similar intensities for both analyte and standard. This similarity helps minimising the error in measurements. The amount of GB for each extract was calculated from the resultant analyte to standard peak ratio according to the following equation:

$$\text{Amount of GB (extract)} = \frac{MW_{GB}}{MW_{IS}} \times \frac{W_{IS}}{W_{extract}} \times \frac{I_{GB}}{I_{IS}} \times P_{IS}$$

Where :

GB and IS refer to glycine betaïne and the internal standard respectively; MW are the respective molecular weights, W the amount of substance used, I the integrated peak area, P the percentage purity of the internal standard (98%). The number of atoms that gives rise to the measured NMR signals being the same for GB and the internal standard (9 H), they cancel in the equation. The amount of GB in the plant is obtained according to the equation:

Amount in the plant = Amount of GB (extract) x extraction yields. Extraction yields are reported in Table 1. Data are expressed in milligrams per gram (mg (gdw^{-1}) of dry matter of *Spartina* tissue (mean ± standard deviation (SD) of three determinations, Table 2).

Table. 2: Comparison of the GB content in leaves and rhizomes for the two species (mg $(gdw)^{-1}$), mean values ±SD).

Date of collection	*S. anglica* Leaves	*S. anglica* Rhizomes	*S. maritima* Leaves	*S. maritima* Rhizomes
27/01/09	21.94±1.01	7.32±0.55	13.88±0.71	3.09±0.31
26/04/09	23.22±0.75	5.05±0.31	15.51±0.95	4.86±0.32
28/06/10	24.24±1.01	5.57±0.43	18.41±1.01	2.5±0.21
22/08/10	22.93±0.82	4.30±0.24	17.34±1.04	3.4±0.31
08/11/10	26.15±1.04	5.76±0.52	15.46±0.99	2.3±0.32
07/03/11	-	3.41±0.29	16.11±1.03	4.5±0.21
15/05/2011	27.61±1.11	12.69±071	19.43±1.01	2.29±0.18

RESULTS AND DISCUSSION

GB is a zwitterionic quaternary ammonium compound that functions as a compatible solute in many plant species including marine algae and halophilic coastal plants. Greenhouse experiments conducted with *S. patens* (Ait.) Muhl. and *S. alterniflora* specimen from coastal Louisiana, cultivated under sublethal stress conditions have shown the importance of GB as osmoprotectant for the genus *Spartina* (Hester *et al.*, 2001 and ref. therein).

Samples of *S. anglica S. maritima* were collected from January 2009 until March 2011 (Table 1). Aqueous methanolic extracts were prepared from both leave and rhizome tissue. Global extraction yields from *S. anglica* vary in the range 17-23.8% $(gdw)^{-1}$ for the leaves and 20.5-25.3% $(gdw)^{-1}$ for the rhizomes (Table 1). Examination of the values shows a very weak seasonal influence. Comparison with the extraction yields from sample of *S. maritima* collected at the same date and place, shows similar values. All the extracts were systematically analysed by ^{1}H- and ^{13}C-NMR, which give a clear understanding of their metabolic content. All the NMR spectra obtained from the leaves of the two species were dominated by the typical pattern of GB (singlets at δ 3.27 and 3.90 ppm due to the $N^+-(CH_3)_3$ and methylene resonances respectively, Fig. 2 a, b). In addition, the presence of GB was confirmed by LC/MS (ESIMS m/z: 118 $[M+1]^+$). Along with GB, an accumulation of sucrose was detected in the rhizomes (comparison with standard).

Quantitative determination of the GB content was performed by ^{1}H NMR with internal standard (see Materials and Methods for details). This fast, non-destructive technique with minimal sample preparation has proved to be useful for quantification of individual components in crude extracts without the need for fractionation or isolation procedures and can compete with or even surpass chromatographic validation based on molecular analysis (Pauli *et al.*, 2012; Nuissier *et al.*, 2008). Assignments of the NMR resonances detected in the one-dimensional (1D) spectra were supported by the comparison of chemical shifts with those of authentic standards. Results are reported in Table 2. The GB content in *S. anglica* varies in the range 21.94-27.61 mg $(gdw)^{-1}$ for the leaves and 3.41-12.69 mg $(gdw)^{-1}$ for the rhizomes. The variation does not seem to be influenced by the season, which is also the case for the native *S. maritima*. However, values for this species are weaker varying in the range 13.88-19.43 mg $(gdw)^{-1}$ for the leaves and 2.3-4.86 mg $(gdw)^{-1}$ for the rhizomes. The relatively low content of the rhizomes is in agreement with the fact that, like for others plants, GB is probably synthesized in leaves and transported to roots (Hanson et al, 1985; Beiss, 1994).

During winter, it is frequent that *S. anglica* leaves decay and disappeared, after periods of severe nightly frost. However, the plant restarts growth from the rhizomes on the next spring. *S. maritima* is not affected by this phenomenon. For this reason, it was not possible to collect leaves of *S. anglica* on March 7th 2011. It is interesting to note that the highest GB content in *S. anglica* (27.60 (leaves) and 12.69 mg $(gdw)^{-1}$ (rhizomes)) were observed with the samples collected on May 2011 following the dieback period.

This is the first long-term monitoring of the GB content for populations of *Spartina* species growing in the mudflat. Some data are available for *Spartina* under cultivation in laboratory. In particular, the effects of nitrogen supply and salinity on GB concentrations in leaves of *S. anglica* grown in greenhouse has been reported (Mulholland and Otte, 2001, 2002). The value reported by the authors for the leaves of the control (18.6 mg $(gdw)^{-1}$) is close to our lowest value. A GB content of 25.9 mg $(gdw)^{-1}$ has also been reported for a sample of *S. townsendii* (Adrian-Romero *et al.*, 1998).

The accumulation of GB in *S. anglica* tissues represents an economical value. Indeed, GB is a small, fascinating molecule, which presents a number of interesting biological activities. As a result, GB has led to a wide field of uses in industry and agriculture for various purposes. A multitude of applications have been described and patented. To give an exhaustive list of the related references would go beyond the scope of this article, and only some typical examples are listed here below.

GB is an important cofactor in methylation, a process that occurs in every cell of mammals to synthesize and donate methyl groups (CH_3) for other processes in the body, including the synthesis of neurotransmitters or melatonin. GB has been shown to protect internal organs, improve vascular and cancer risk factors, and enhance performance (Ueland, 2011). As results, a multitude of therapeutic combinations comprising GB have been patented.

GB has also found applications as a diet supplements in the treatment of liver disorders, for hyperkalemia, for homocystinuria, and for gastrointestinal disturbances (Lipmann, 1993). GB has been used in for >50 years, and its application in human nutrition has recently been reviewed (Craig, 2004). In addition, GB is used as an ingredient of personal-care formulations (Rigano et al. 2000) and many products have been patented. It has been demonstrated that exogenous application of GB improves the growth and survival rate of plants under a variety of stresses and have a role in aiding plants to resist attack by pathogens (see as examples Giri, 2011; Wu *et al.*, 1997; Tyihak *et al.*, 2002). This led to a wide range of applications in agriculture (see as example: Amhad *et al.*, 2013; Miles *et al.*, 2003; Sanchez et *al.*, 2011).

Members of the Chenopodiaceae, such as sugar beet and spinach, accumulate GB. GB is industrially produced from sugar beet molasses (Nam Eng, 2003).

The GB content of sugar beet varies in the range 1.14-2.97 mg.g-1 (Zeisel *et al.*, 2003), but it is not clear if these values are expressed on a fresh or dry weight basis, which makes direct comparisons with our results difficult. However, Adrian-Romero *et al.* (1998) determined the GB content of *S. townsendii* and sugar beet in the same study. The value reported for *S. townsendii* (25.9 mg $(gdw)^{-1}$) falls in the range of ours for *S. anglica* (21.94-27.61 mg $(gdw)^{-1}$), and about three time higher the content of sugar beet (7.9 mg $(gdw)^{-1}$). This last value is in agreement with the 7.8 mg $(gdw)^{-1}$ concentration reported by Beiss (1994) for the four-year average GB content of sugar beet. From these data, we can conclude that *S. anglica* is an excellent and competitive accumulator of GB.

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