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The Effect of Growth Environment and Salinity on Lipid Production and Composition of *Salicornia virginica*

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Abstract

Finding a viable and sustainable source of renewable energy is a global task. Biofuels as a renewable energy source can potentially be a viable option for sustaining long-term energy needs. Biodiesel from halophytes shows great promise due to their ability to serve not only as a fuel source, but a food source as well. Halophytes are one of the few biomass plant species that can tolerate a wide range of saline conditions. We investigate the feasibility of using the halophyte, *Salicornia virginica* as a biofuel source by conducting a series of experiments utilizing various growth and salinity conditions. The goal is to determine if the saline content of *Salicornia virginica* in our indoor growth versus outdoor growth conditions has an influence on lipid recovery and total biomass composition. We focused on using standard lipid extraction protocols and characterization methods to evaluate 12 *Salicornia virginica* samples under six saline values ranging from freshwater to seawater and two growth conditions. The overall goal is to develop an optimal lipid extraction protocol for *Salicornia virginica* and potentially apply this protocol to halophytes in general.

Introduction

Accepting the fact that conventional sources of inexpensive fuel and energy are rapidly depleting and cannot be renewed, identifying renewable energy sources is a global task that will require innovative thinking and methods. Biofuels are a viable option for sustaining long-term renewable energy needs because they satisfy the criteria to be considered green ecologically friendly energy sources—halophytes are alternative, renewable and sustainable (Refs. 1, 2, 3, and 4). More specifically, developing alternative energy needs that do not rely on freshwater, compete with food crops or use arable land are increasingly important due to increased world demand on these resources. Halophytes are a viable option for renewable energy biomass because they can potentially be used as a fuel or food source and do not rely on freshwater or compete with existing land that conventional food crops utilize (Refs. 5, 6, 7, and 8).

A halophyte is any plant (macro or micro) that is able to grow and thrive in salt-water environments or brackish-water such as salt marshes, seas coasts and deserts. The world flora lists approximately 885 species of halophytic angiosperms distributed over 250 genera (Ref. 8). Less than 2 percent of plant species are halophytes. Halophytes have a variety of advantages over traditional crops used for biofuel.

* NASA Intern with Lewis Educational and Research Collaborative Internship Project

They minimally impact the scarce and rapidly depleting resources of fresh water and arable land and are capable of thriving in a variety of environments. Unlike corn or soybeans, halophytes are not traditionally used as a food crop and their use as a source of biofuel will have minimal economic or sociological consequences. Approximately 50 species of seed-bearing halophytes are potential sources of lipids, which are the plant product extracted for biofuel. Glenn (Ref. 5) and Hodges (Ref. 9) have tested the feasibility of using seawater agriculture to grow halophytes and found that halophytes grow well under these conditions in desert or sandy soil environments. It is likely that most new halophyte applications will be inland as most halophytes have reduced productivity at very high salinities (Refs. 10 and 11), along with other considerations, such as more traditional farming practices and population distribution.

Halophytes are applicable in developing subtropical desert countries with limited freshwater and growing populations. One of the most urgent global problems is finding enough fresh-water and arable-land to support the world's food needs. The United Nations Food and Agriculture Organization (Ref. 12) estimates that an additional 200 million hectares of new cropland will be needed over the next 30 years just to feed the burgeoning populations of the tropics and subtropics. However, only 93 million hectares are currently available and easily accessible. Halophytes do not require traditional arable land and are potentially a valuable cash crop and an elegant solution to increasing demand for food and livestock feed. An analysis (Ref. 13) performed at NASA Glenn Research Center (GRC) projects that advanced halophyte agriculture that utilizes available arid and semi-arid land can provide for the projected world energy demand. Additionally, halophyte seeds have higher lipid percentages than traditionally used oilseeds and potentially the maximum amount of oil produced per acre is much greater for halophytic plots (Refs. 1 and 14) with comparable total biomass.

The halophyte *Salicornia virginica* naturally occurs in a wide distribution and is adaptable to a variety of salinities. *Salicornia virginica* (also known as *Salicornia depressa*) is a perennial, coastal, salt-marsh halophyte native to Florida and California and found as far north as Alaska. *Salicornia virginica* is the dominant mid-elevation plant species in salt-marshes and is a typical first-colonizer. An appealing aspect in maintaining an area of *Salicornia virginica* is that possible competitors cannot tolerate salt ions (i.e., high salinities) as effectively as *Salicornia virginica* and unwanted or invasive species can be managed through controlled soil salinity (Ref. 14). *Salicornia virginica* has the ability and is known to respond to increased salinity levels by decreasing root conductivity and increasing salt uptake, but its growth rates are minimally affected by salinity (Ref. 15). Unlike glycophitic plants, *Salicornia virginica* can reversibly change its root characteristics to adapt to different levels of salinity. Other research supports that the CO_2 uptake, stomata opening and other indicators of photosynthetic activity of *Salicornia virginica* are affected by salinity to a lesser degree than other plants (Ref. 16). Figure 1 to Figure 6 show various habitats of *Salicornia virginica*

The primary goal of this study was to identity the influence of (1) the saline content of the growth media and (2) controlled versus natural environment on extraction recovery, lipid content, and lipid distribution of *Salicornia virginica*, with attention to total biomass (e.g., Figure 1 to Figure 6). GRC provided NETL-ORD with 12 *Salicornia virginica* specimens representing a matrix of six salinity values that were either collected from a controlled indoor laboratory or an outside environment (NASAs GreenLab Research Facility (GreenLab)).

NETL performed two different lipid extraction methods, chosen based on previous evaluations of several methods, and determined the amount of lipids produced by each plant based on gravimetric recovery. Each sample of lipid extract was subsequently characterized by GC-MS, with mass spec library matches being used to identifying the major species present in the extracts. Extracts are currently being further characterized for concentration of major species using GC-FID.

Figure 1.—*Salicornia virginica* (Florida).

Figure 2.—*Salicornia virginica* (Florida).

Figure 3.—*Salicornia virginica* seedlings (GreenLab).

Figure 4.—*Salicornia virginica* (Indoor Biofuels Lab)

Figure 5.—*Salicornia virginica* (GreenLab)

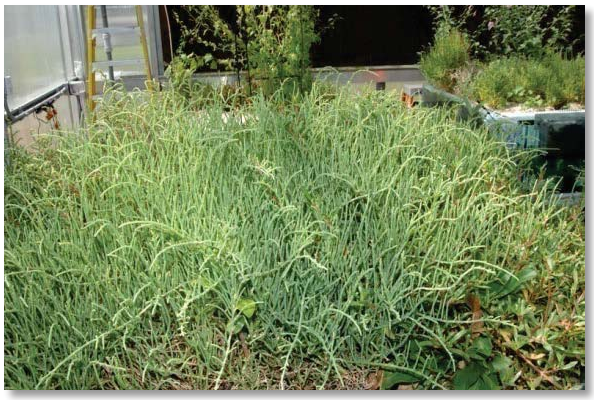

Figure 6.—*Salicornia virginica* (GreenLab)

Method

Samples of *Salicornia virginica* grown both indoors and outdoors (GreenLab) under six different saline conditions ranging from freshwater to natural seawater were collected by GRC were delivered to NETL. Specimens grown in the indoor, climate-controlled laboratory were grown from seeds obtained from a commercial seed production company. Each plant was raised exclusively in a media of specific salinity for its entire life cycle. Specimens comprising the GreenLab population were gathered from a population of wild *Salicornia* in the Florida Keys and acclimated to each level of salinity in the Indoor lab. Biomass was gathered when plants were mature and seeds were then processed to determine lipid composition and content.

The following protocol was used to process samples:

Biomass Preprocessing

1.1 Leaf tissues were separated from the stem of the plant and placed in sealed plastic bags at room temperature for storage.
1.2 To determine the moisture content of *Salicornia*, samples were taken from the initial stock and dried to constant weight.
1.3 To account for moisture lost during extraction, a duplicate portion from the sample being extracted was massed and dried to constant weight.

Hexane-Based Extraction

2.1 5 g of leaf tissue was massed and mixed with 1.5 mL 2M HCl.
2.2 Samples were homogenized by hand-chopping with a sharp metal spatula for 2 min.
2.3 The mixture was placed in a 60 °C water bath for 60 min.
2.4 8 mL of hexane was added and the extraction tubes shaken.
2.5 The mixture was placed in water bath at 60 °C for 15 min. and mixed periodically by shaking.
2.6 The mixture was centrifuged and the supernatant removed using a glass pipette;
2.7 The pellet was re-extracted twice with fresh 8-mL aliquots of hexane.
2.8 Supernatants were combined and dried completely under a low flow of nitrogen. Lipids manifested as an opaque residue on the bottom of each tube.
2.9 Residues were completely redissolved in 2:1 (v:v) mixture of chloroform:methanol

Methanol-Based Extraction

3.1 5 g of plant tissues were weighed and mixed with 5 mL of 0.8 percent HCl in methanol.
3.2 Samples were homogenized by hand-chopping with a sharp metal spatula for 2 min.
3.3 The mixture was placed in water bath at 60 °C for 15 min., and mixed periodically by shaking.
3.4 The mixture was centrifuged and the supernatant removed using a glass pipette;
3.5 The pellet was re-extracted twice with fresh 5-mL aliquots of 0.8% hexane
3.5 All supernatants were combined for post-extraction workup.
3.6 Combined supernatants were dried under a low flow of nitrogen.
3.7 Residues were redissolved in 2:1 (v:v) mixture of chloroform:methanol

***Note**. A significant portion of extract did not redissolve in chloroform:methanol, suggesting that the residue contained appreciable quantities of co-extracted, nonlipid material. Recoveries based solely on gravimetric determination show extremely high bias due to co-extracted material. Further filtration of the redissolved sample was required to correct for fine insoluble particulate matter (most biomass processing requires precleansing of raw-oils).

Extract Species Characterization

Following the redissolution of the residues in 2:1 $MeCl_2$: MeOH (CH3OH), the fractions were analyzed by both GC-MS (for primary characterization) and GC-FID (for quantification of selected species). The GC-MS analysis proceeded according to the parameters outlined in Table I. After obtaining an appropriate chromatogram, peak identifications were made using Chemstation (Agilent) data processing, by searching against a spectral library (NIST), and by evaluating the quality of the match identified. In some instances, pure compounds were injected to determine their retention time and to provide additional evidence to validate the identification made by mass spectrum library search.

The GC-FID analysis proceeded according to the parameters outlined in Table II. Because preliminary characterization yielded some degree of knowledge as to the species present and their respective retention times, calibration standards for many of the target components (free fatty acids, esters, sterols, etc.) were prepared and detector response was determined at different concentrations. After obtaining an appropriate calibration for each individual target species, samples of redissolved residue were analyzed and the concentration of the species was calculated. The masses of each individual species were summed, and compared to the mass of the extract residue.

TABLE I.—RELEVANT PARAMETERS FOR GC-MS CHARACTERIZATION OF REDISSOLVED EXTRACT RESIDUES.
[Chromatography was done using Agilent 6890 GC-Agilent 5973 MSD.]

Inlet	Temperature = 360 °C Split Ratio = 25:1 Injection volume = 1.0 µL (autosampler) Constant flow at 0.8 mL/min.
Column	Zebron Inferno ZD-1HT (Phenomenex) 100% dimethyl polysiloxane 30- by 0.25-mm ID, 0.25 µm film thickness
Oven	Initial temperature = 40 °C (hold 0.5 min.) Ramp from 40 to 360 °C at 6 °C/min. Final temperature = 360 °C (hold 10 min.)
Detector	Transfer line temperature = 360 °C

TABLE II.—RELEVANT PARAMETERS FOR GC-FID CHARACTERIZATIONOF REDISSOLVED EXTRACT RESIDUES.
[Chromatography was done using Agilent 6890 GC-FID.]

Inlet	Temperature = 360 °C Split Ratio = 25:1 Injection volume = 1.0 µL (autosampler) Constant flow at 1.2 mL/min
Column	Zebron Inferno ZD-1HT (Phenomenex) 100% dimethyl polysiloxane 30- by 0.25-mm ID, 0.25 µm film thickness
Oven	Initial temperature = 40 °C (hold 0.5 min.) Ramp from 40 to 360 °C at 6 °C/min. Final temperature = 360 °C (hold 10 min.)
Detector	Temperature = 360 °C

Results

Gravimetric Recoveries

Gravimetric lipid recoveries of the *Salicornia virginica* specimens evaluated in this study are reported in Table III and illustrated in Figure 7, using the two aforementioned protocols.

Extract Composition

Hexane-HCl Extraction

The extracted materials from the 12 *Salicornia virginica* samples were characterized in duplicate and averaged. Residue extracted using the hexane-HCl based extraction method qualitatively exhibited the same constituent lipid components, detailed in Table IV and Figure 7. The major species identified in the lipid extracts were hexadecanoic acid (palmitic acid), octadecanoic acid (stearic acid) and C18:1 to C18:3 unsaturated acids (oleic, linoleic and linolenic acids). Additional species identified were ergostanol, stigmasterol, sitosterol, and stigmastadiene isomers. Appreciable amounts of phytol were identified as well as carotenoid-based degradation products. The phytols presumably result from the breakdown of chlorophyll in the harsh, high-temperature conditions of the GC injector (360 °C). In general, the hexane-HCl extraction yields a cleaner lipid fraction, as most of the polar co-extracted species seen with the MeOH-HCl extraction are excluded.

TABLE III.—GRAVIMETRIC LIPID RECOVERY[a] FOR ALL SAMPLES USING THREE EXTRACTION METHODS

Growth environment	Salinity of growth media, [b]TSG	Hexane + 2M HCl	Methanol + 2M HCl	[c] Methanol + 2M HCl
Indoor	1.000	3.2 ± 0.5	61.1 ± 0.5	26.9 ± 5.5
Indoor	1.005	4.1 ± 0.1	52.9 ± 9.0	17.5 ± 5.3
Indoor	1.010	3.6 ± 0.1	64.6 ± 3.4	23.5 ± 1.0
Indoor	1.015	2.1 ± 0.1	72.9 ± 3.2	25.5 ± 3.8
Indoor	1.020	2.4 ± 0.1	64.5 ± 2.8	21.2 ± 0.3
Indoor	1.025	2.7 ± 0.1	92.4 ± 5.7	37.6 ± 4.4
GreenLab	1.000	2.8 ± 0.1	62.4 ± 0.8	23.2 ± 2.7
GreenLab	1.005	3.4 ± 0.2	63.7 ± 6.1	19.5 ± 2.1
GreenLab	1.010	2.4 ± 0.0	49.5 ± 3.1	15.0 ± 0.8
GreenLab	1.015	2.0 ± 0.4	55.8 ± 0.5	31.1 ± 13.0
GreenLab	1.020	2.1 ± 0.1	91.8 ± 21.2	26.4 ± 4.0
GreenLab	1.025	3.1 ± 0.2	57.7 ± 2.7	19.5 ± 0.6

[a] Recoveries are the average of two replicate samples, based on the entire residue remaining after extraction compared to the dry weight of starting material.
[b] TSG: True Specific Gravity, as measured with a refractometer and salinity meter.
[c.] Recoveries corrected for co-extracted material that did not redissolve in chloroform:methanol.

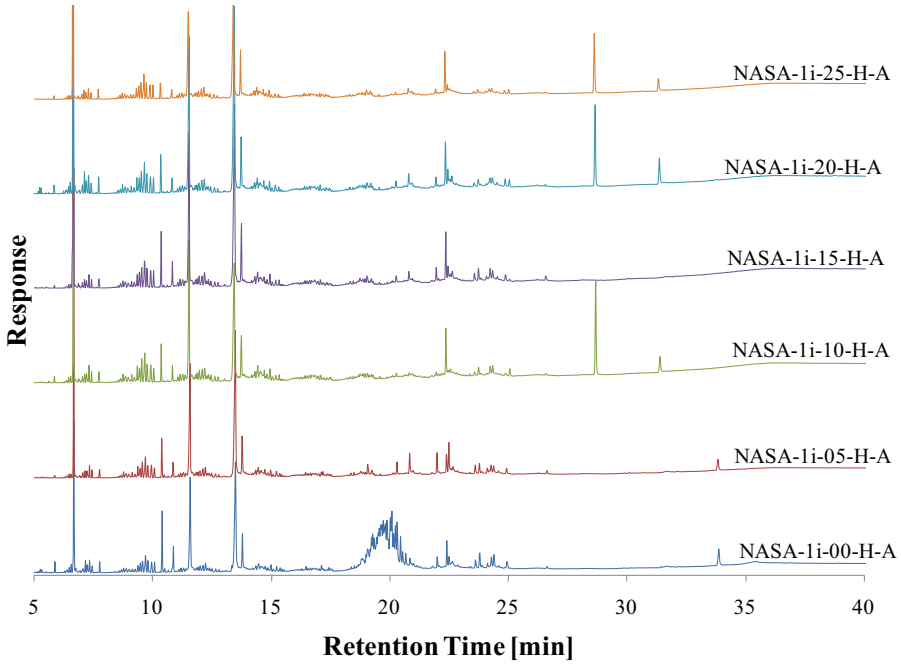

Figure 7.—Typical GC-MS chromatograms for the extracted lipid content using the hexane-HCl solvent for the Salicornia Virginica samples of focus in this study.

TABLE IV.—QUALITATIVE SPECIATION OF THE LIPID CONTENT OF THE *SALICORNIA VIRGINICA* USING A HEXANE-HCl EXTRACTION

Retention time, min.	Chemical name	Common name	Notation
6.6	Dodecane		
10.4	2,6-di-tertbutyl-4-methylphenol	BHT	
11.5	Hexadecanoic acid	Palmitic acid	C16:0 acid
13.5	*cis*-9,*cis*-12-Octadecadienoic acid	Linoleic acid	C18:2 $\Delta^{9,12}$acid
13.6	*cis,cis,cis*-9,12,15-Octadecatrienoic acid	Linolenic acid	C18:3 $\Delta^{9,12,15}$ acid
13.7	*cis*-9-Octadecenoic acid	Oleic acid	C18:1 Δ^{9} acid
13.8	Octadecanoic acid	Stearic acid	C18:0 acid
22.4	Bis (2,2,6,6,-tetramethyl-4-piperidyl) sebacate	Tinuvin 770	
22.48	Tocopherol	Vitamin E	sterol
23.60	24α-Ethyl-5α-cholestan-3β-ol	Stigmastanol	sterol
24.4	24-Ethyl-4,22E-cholestadien	Stigmastadien	sterol
24.45	5-α-ergostan-β-3-ol	Ergostanol	sterol
28.6		Unknown ID	
31.3		Unknown ID	
33.8		Glycolipid	

MeOH-HCl Extraction

The extracted materials from the 12 *Salicornia virginica* samples were characterized in duplicate for chemical speciation. As expected, the MeOH-HCl extraction yields a material in which most of the lipid material consisted of methyl esters of C16 and C18 fatty acids, along with trace amount of free fatty acids

of these carbon lengths, detailed in Table V and illustrated in Figure 8. Although more difficult to see, the acidic methanol extracts appear to display varying ratios of the C16 acid to C18 acids when the observed intensities of both the free acids and the methyl-esters of those acids are considered as shown in Figure 9.

TABLE V.—QUALITATIVE SPECIATION OF THE LIPID CONTENT OF THE *SALICORNIA VIRGINICA* USING A MeOH-HCl EXTRACTION

Retention time, min.	Chemical name	Common name
6.6	dodecane	
10.4	2,6-di-tertbutyl-4-methylphenol	BHT
10.18	Methyl *cis*-9-Hexadecenoate	methylpalmitoleate
11.17	Methyl hexadecanoate	methylpalmitate
11.3	*cis*-9-Hexadecenoic acid	Palmitoleic acid
11.52	Hexadecanoic acid	Palmitic acid
12.86	Methyl *cis*-9,*cis*-12-Octadecadienoate	Methyl linoleate
12.95	Methyl *cis,cis,cis*-9,12,15-Octadecatrienoate	Methyl linolenate
13.05	Methyl *cis*-9-Octadecenoate	Methyl oleate
13.3	*Cis*-9,*cis*-12-Octadecadienoic acid	Linoleic
13.4	*cis,cis,cis*-9,12,15-Octadecatrienoic acid	Linolenic
13.45	Methyl octadecanoate	methylstearate
13.5	*cis*-9-Octadecenoic acid	Oleic acid
13.7	Octadecanoic acid	Stearic acid
22.48	Tocopherol	Vitamin E
23.60	24α-Ethyl-5α-cholestan-3β-ol	Stigmastanol
24.4	24-Ethyl-4,22E-cholestadien	Stigmastadien
24.45	5-α-ergostan-β-3-ol	Ergostanol

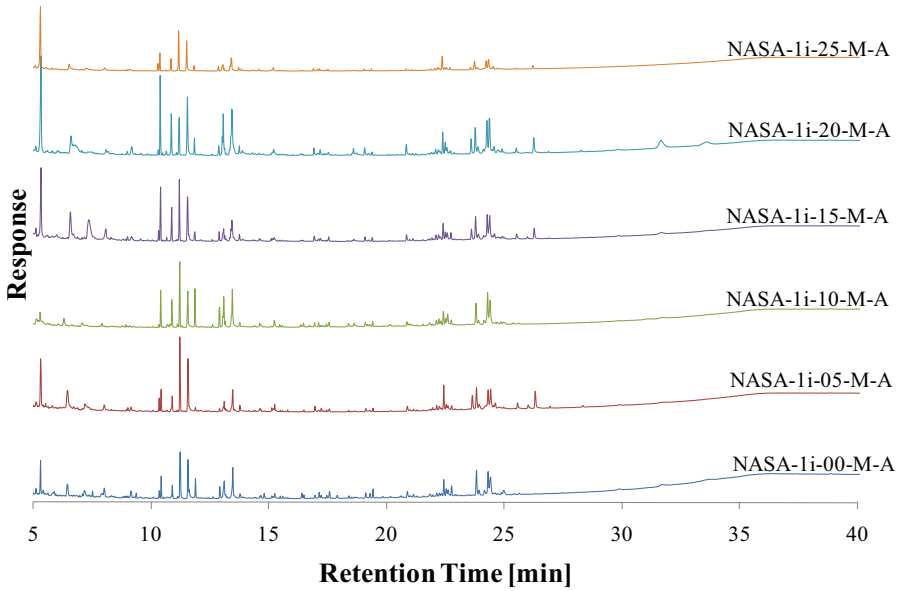

Figure 8.—Typical GC-MS chromatograms for the extracted lipid content using the MeOH-HCl solvent for *Salicornia virginica* samples.

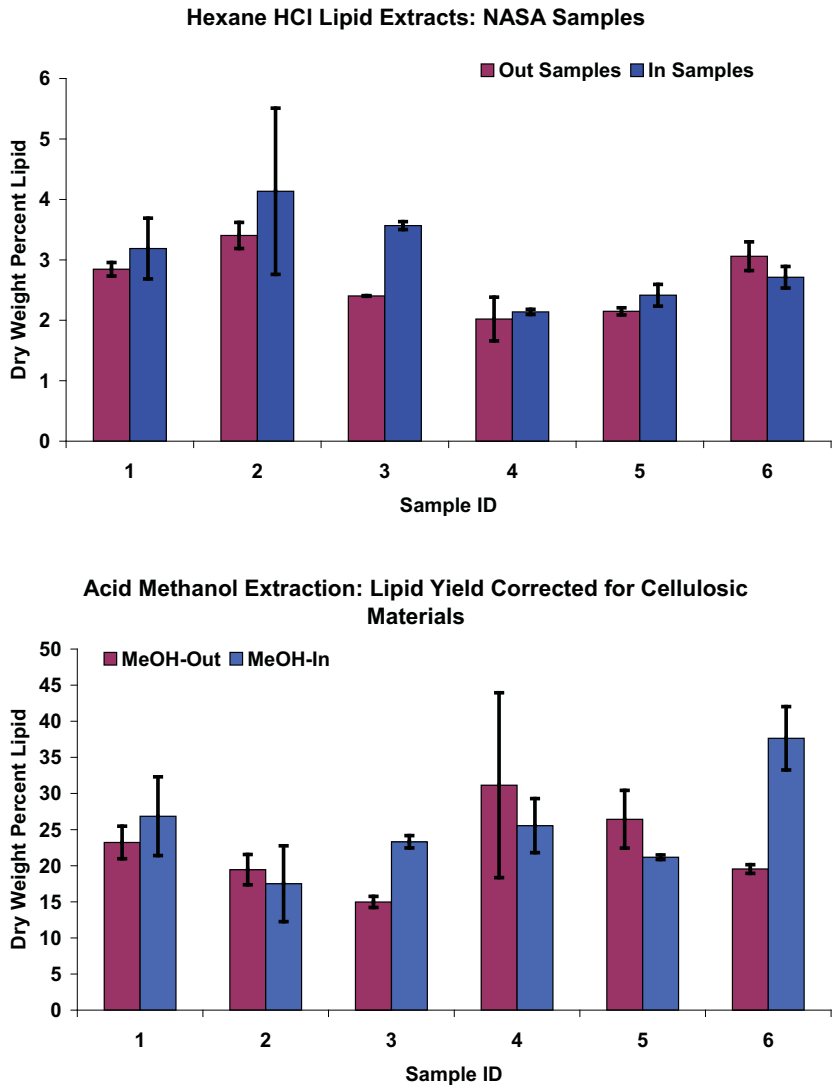

Figure 9.—Comparison of extract yields based on gravimetric methods as a function of growth environment and extraction solvent.

Growth Environment

Comparisons of the extract yields for the indoor and outdoor samples as a function of extraction solvent are illustrated in Figure 9. On average, the extracted material for the hexane-based extractions appeared to be slightly higher for the samples grown in the Indoor as compared to the samples grown in the GreenLab. There is not a large statistical significance to this difference. One important note is that the MeOH-based extracts were found to contain significant amounts of insoluble cellulosic residues and required further cleaning prior to final gravimetric yield determination. Despite our best efforts, the cellulosic residues, as well as possible soluble protein fractions, most likely cloud any trends in the MeOH-based extractions.

Discussion

As mentioned previously, the determination of lipid based solely on the amount of extracted material can lead to inaccurate assumptions about lipid production as well as lipid composition. Our initial attempts at calibrating the FID response for the dozens of individual species seen in the *Salicornia*

virginica extracts were met with limited success. The primary difficulty is that not only do the various species respond differently in the FID, but also the various chain lengths within the same species lead to different responses. Moreover, it is difficult to detect species such as triacylglycerides using gas chromatography, primarily due to poor volatility. We are currently exploring methods for improving our quantitative analysis of the extracts so that in the future we can provide a lipid-specific quantity, including species such as free fatty acids, esters, and glycerides, while excluding any extraneous materials such as chlorophyll, glycerol, etc. Traditional derivatization methods, which convert all species to methyl esters, obscure the compositional makeup of the original extracts. This compositional makeup may be critical in determining the optimum reaction pathways for converting the raw extract into useful fuels. Therefore, we continue to pursue methods whereby the original biomass extract composition can be established and a lipid-specific quantization of biomass oils produced can be determined.

Conclusions

On the surface, it appears that extraction of lipids from *Salicornia virginica* using a polar solvent such as methanol provides the most amount of material, and establishes a gravimetric lipid production at approximately 30 percent (dry weight). However, from the compositional analysis, it is obvious that this extracted material contains significant portions of species that are not typically considered to be lipids, and so gravimetric lipid content is artificially high. Moreover, the species that dominate this fraction are methyl esters of C16 and C18 fatty acids. On the other hand, hexane extraction appeared to remove significantly less lipid based on mass. Compositional analysis shows that acidic hexane provides a much cleaner fraction, composed primarily of free fatty acids.

An important finding is that when using the extraction methods in this study, it is apparent that growth conditions did not influence extraction recoveries, lipid content or lipid distribution, within statistical error. This is significant as we move to expand our study to actual field trials around the world under varying salinity conditions.

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