Propagation of *Chara vulgaris* Using Sediment Seed Bank Oospores and Vegetative Biomass

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For Wisutec

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SUMMARY

Methods have been developed whereby viable oospores of the charophyte *Chara vulgaris*‘ are routinely collected, concentrated, and tested for viability. High redox conditions (e.g. shallow tap water) at 20^{o}C support oospore germination. Germination does not occur at 4^{o}C.

Four of five source populations tested could serve as a source of oospores for systems such as Poehla, while variability in oospore germination trials precludes the fifth site (Langmuir) as a reliable source.

Oospore concentrate drying and storage at 4^{o}C has been proven in the 2003 germination trial as an effective way to handle and store oospores in a viable state for the long term (1 year or more).

Specific configurations of substrates which support both the germination of oospores and the rapid growth of sporelings have been tested. The results indicate that a 1 cm layer of washed sand over nutrient-enriched dried clay promotes germination and supports rapid sporling growth.

Chara vulgaris growth rates in the laboratory were determined. In aquarium cultures growth rates of 0.71 to 1.58 gdw.m^{-2}.d^{-1} were measured.

1.0 INTRODUCTION

A simple, reliable technique for establishing *Chara* populations in biological treatment systems is being developed. This research is examining the feasibility of collecting, storing and seeding of *Chara* oospores, or seeds, as a means of introducing populations to engineered biological polishing ponds.

The suitability of available substrates in terms of supporting rapid growth and development of the plants must also be assessed, since there is growing evidence that *Chara* species may largely depend on nutrients only available in the sediment phase, and extract these via their rhizoids in the establishment phase of the population.

Growth rates and biomass production by *Chara* species are also being determined in the lab as input data required for assessing the efficacy of ^{226}Ra removal by *Chara* populations in the planned Poehla treatment system.

2.0 OBJECTIVES

To provide insight for *in vitro* germination of *Chara vulgaris* from oospores and establishment from vegetative biomass in relationship to storage application and sediment type. Our studies are comprised of laboratory preliminary results and germination trial repeats from oospores concentrated in 2001 and shipped to Wisutec in February 2003.

This report assesses the pattern of germination of *C. vulgaris* from seed bank sediment oospores *in vitro*, under wet/ dry storage conditions of varying duration and from biomass.

3.0 METHODS AND MATERIALS

Five sites in Ontario were selected for the collection of sediment on which *Chara vulgaris* populations existed. In November of 2001 sediment was collected from: **Langmuir** - a pond containing tailings generated from nickel extraction (48 ^{o}35' N, 81 ^{o}52' W) , Little Pearl Lake (**LPL**), **Schumacher 1** and **2** from gold mining operations (all from northern Ontario), and **St. Clair**, a wetland pond adjacent to L. St. Clair receiving natural and agricultural run-off in southwest Ontario (42 ^{o} 22'N, 82^{o} 22'W). Table 1 provides some physical characteristics associated with each site.

Table 1: Physical Charateristics of Sediments and Water Chemistry (sampled October 25-27, 2002) for Chara Oospore Concentration

Site Location			Area	Depth	Description of Sediment			Wet Density g/mL	Moisture %	Chemistry of water above sediment		
Label	Whole Name	Location	m^2	m	colour	smell	texture			pH	Eh (mv)	Cond (uS/cm)
St. Clair	St. Clair	No Tailings - Chatham Ont.	1000	0.6	dark brown mixed with some shining spots	organic smell	silt mixed with peat, lots of decomposed organics	1.21	56.3	8.2	N/A	479
Sch 1	Schumacher 1	wetlands beside tailings	2700	0.25-0.5*	dark mixed with lots of shining spots	very strong H_2S smell	silt, lots of straw, sponge like sediment	1.03	82.7	6.8	470	1330
Sch 2	Schumacher 2	decant tower receiving pond	4500	0.4	dark	strong H_2S smell	fine silt, some staw, sponge like sediment	1.24	62.7	7.24	488	2480
Langmuir	Langmuir	tailings pond	45500	0.5-1.9	dark mixed with lots of shining spots	very strong H_2S smell	silt, full of straw	0.99	80.4	7.73	1227	544
LPL	Little Pearl Lake	tailings pond mine water receiver	22500	0.5-2.7	ink dark	very strong H_2S smell	peat, very fine, some straw, sponge like sediment	1.04	75.7	7.44	272	2850

* 0.25m in May 2003

The oospores have been accumulating in the sediment from parent plants from previous growing seasons, thus the age distributions of the oospore seed bank populations supplied to Wisutec are not known.

For instance, Schumacher 1 and Schumacher 2 populations are both located at the base of once active gold tailings dams, and likely established only after active mining ceased. Similarly, *Chara* was not likely in the Langmuir tailings pond during the mine's life, and only since has the population proliferated.

Little Pearl Lake was dredged in the late 1980s, and this *Chara* population is less than 23 years old. Meanwhile, the St. Clair *Chara* population is at the base of a man-made berm of unknown age.

3.1 Oospore Collection and Concentration

Sediment was collected from the top 15 cm of each site using a shovel and placed into 20 L plastic pails with lids for transport to the laboratory. These were stored in the dark at 8 to 10^{o}C for 21 days prior to the first germination trial and for four months prior to concentrating the oospores by sieving and cycloning, drying and long-term storage at 4^{o}C.

Oospores were concentrated by passing the sediment mixture through a 1.0 mm screen, discarding materials > 1mm. The oospores were then retained on a 200 um screen, with smaller particles passing through the screen. This concentrate, comprised of 200 um to 1000 um particles, was placed in the 200 um screen and tap water added in cyclone motion to remove lighter particles, until only oospores and other dense particles (e.g. sand) remained. These were dried on paper at room temperature for 2 days, and stored into glass-jars covered with aluminum-foil in the fridge at 6^{o}C. The oospore concentrate has been supplied to Wisutec in February 2003 for work in the Poehla system.

3.2 Germination from Oospores

Two different germination trials were carried out in 2001 and 2002 / 2003. The 2001 trial's oospores remained wet in their original sediment from the time of field collection until the end of the germination trial, while the June 2002 and April 2003 germination trials used oospore concentrate dried in February 2002 and stored at 4^{o}C until experiment set-up.

Germination differences among storage type and duration were accounted for using SPSS statistical package, ANOVA and Paired Samples T-tests. For the 2001 (wet) and 2003 (dry) experiments, we examined whether a significant difference between the overall - accumulative germination rates exists for a given population subjected to different storage conditions.

Similarly, for the 2002 (dry) and 2003 (dry) experiments, significant differences between the overall germination rates were examined for all populations for the 1 year storage duration. Table 2 provides a summary of the methods employed and results for the germination from oospore trials.

Table 2: Summary of 2001, 2002 and 2003 germination trial methods and results.							
Year	Spp.	Date Collected	Stratification Start	Date Refined	Storage Conditions	Methods	Results
2001	*C.v.*	21-Oct-01	21-Oct-01	11-Nov-01	Wet 8-10°C, 21 d sediment storage.	Five populations tested; aerobic refine; hand-picked wet oospores incubated in petri dishes with water in light.	17 to 49 % germination in 21 day stratified samples.
		21-Oct-01	21-Oct-01	11-Nov-01	Wet 8-10°C, 21 d storage; 4°C, 15 d isolated in petri dishes.		21 to 43 % germination in 21 +15 day stratified - E_7 high in all petri dishes, ranging from 442 to 503 mV.
2002	*C.v.*	21-Oct-01	2-Mar-02	28-Feb-02	Oospores dried and stored at 4°C for 97 days in fridge	Five populations tested; dried oospores incubated in petri dishes with water in light.	2% to 35 % germination following 97 days stratification as dried oospores. Four of five populations %germination decreased w.r.t. 2001 Exp't. Langmuir declined from
2003	*C.v.*	21-Oct-01	2-Mar-02	28-Feb-02	Oospores dried and stored at 4°C for 376 days in fridge	Five populations tested; dried oospores incubated in petri dishes with water in light.	3% to 42 % germination following 376 days stratification as dried oospores. Two of five populations %germination decreased w.r.t. 2001 and 2002 Exp'ts. Two other populations %gemination increased w.r.t. 2002 Exp't. Langmuir remained at 3%.

3.2.1 2001 Trial (Wet Storage)

Sediment samples were stored in closed containers in the dark at 8 to 10°C for 21 days since collection in the field in late October, 2001and prior to sampling and refining oospore concentrates using 4°C oxygenated tap water.

Petri dishes containing 30 ml of degassed tap water were prepared. Twenty-five oospores were selected from wet oospore concentrate from each population and added to six Petri dishes. Three Petri dishes were placed in an illuminated refrigerator set at 4°C. The three remaining Petri plates were incubated at 20°C under a fluorescent light bank.

Oospores in petri dishes incubated in a fridge for 15 days were examined under a stereo microscope on days 6, 10 and 15. Since less than 1 % of the oospores (1/396) germinated over the 15 days, these Petri dishes were transferred to 20^{o}C and re-examined 10, 15 and 25 days later. Oospores in Petri plates incubated at 20^{o}C immediately following set-up were examined on days 6, 10, 15, 24, 29 and 39. Each population's water chemistry: pH, redox potential (Eh), Electrical Conductivity (EC) and temperature were measured on the first and last day of incubation.

3.2.2 2002 Trial (Dry Cold Storage for 96 Days)

Oospores in sediment samples from all five population collected in late October 2001 were concentrated, dried and put into 4^{o}C storage in glass jars wrapped with aluminium foil in February, 2002. In June, 2002, five Petri plates containing 30 mL of tap water were set up and 33 to 74 dried oospores were added. These dried oospores had been stored at 4^{o}C for 96 days after refining and drying the concentrate. The Petri plates were placed under fluorescent lights at room temperature with 12 h:12h light: dark. Oospore germination was assessed 12, 20 and 48 days after set-up.

3.2.3 2003 Trial (Dry Cold Storage for 376 days)

Other than the length of the dry cold storage period, the method employed in the 2003 germination trial departs from that of 2002 in the number of *C. vulgaris* oospores added to each Petri dish; about 80% more were added in 2003, and a more frequent germination monitoring program was performed.

Petri dishes containing 30 ml of deionised water were prepared. Fifty to 150 oospores (+/- 5.38 SD) from each population were added to six Petri dishes, and counted three times. All dishes were illuminated from above with florescent light bank (Sylvana 40W

Daylight Deluxe F40/DX) with a 12 hr. light: 12hr dark cycle, at a room temperature of 20^{o}C, incubating for 1.5 months. All dishes (except the 6th – the control) were examined under a stereo microscope every other day during the peak germinating rates and weekly otherwise.

Germination was defined as the emergence of both rhizoids and the protonema. Individual germinated oospores were picked out using a pipette under the stereo microscope, removed upon counting and further germinated separately (see Section 3.5, "Sediment Types and Development"). On the same day, the sixth Petri dish of each population was used for water chemistry (pH, Eh, EC and temperature) measurement.

The pH and redox potential were measured using a Corning 315 pH/ion meter. Redox potential is usually expressed as E_7, in which a correction is made for the change in redox at the pH of the sample to a pH of 7 (Wetzel, 1983). The E_7 , in mV, is calculated as follows:

$$E_7 = (E_{measured} + (241\ mV-(0.66 * (T\ ^{o}C-25^{o}C))) + (pH-7)*58\ mV$$

3.3 *C. vulgaris* Establishment and Growth Rates From Biomass

St. Clair *Chara* biomass grown from oospores in the lab was transferred to an aquarium containing a 7 cm thick layer of nutrient-enriched clay and tap water placed in natural lighting (window sill). *Chara* shoots were held against the sediment using plastic netting weighted with plastic covered wire rope on January 30th 2003. The aquarium received natural light and had an average temperature of 20^{o}C.

On April 16, after 76 days, the *Chara* biomass was trimmed back, leaving only 2 cm of still-rooted emergent biomass above the sediment. The water was replaced with new tap water. Half of the harvested biomass (11.5 g wet wt.) was transplanted into a separate aquarium under florescent light at room temperature with tap water for

biomass evaluation. Shoots 2 to 8 cm long were held by plastic mesh and wire against a 3 cm thick nutrient-enriched clay layer covered with a 1 cm layer of washed masonry washed sand.

On June 3rd, the harvesting process was repeated, followed by reweighing of wet and dry biomass. Water chemistry (pH, Eh, EC and temperature) was recorded biweekly. *Chara* growth has been monitored by a Logitech digital camera connected to ImageStudio 2002, set to a 30 minute interval snap shot creating three sequels: February 6th to April 16th 2003, April 16th to May 13th 2003, June 3 to July 3, 2003.

3.4 *C. vulgaris* Germination Projection from Oospore Density

Dried oospores were directly seeded in an aquarium in the lab in order to examine whether oospores will germinate and rapidly develop into large plants if the correct substrate, in terms of supporting both oospore germination and sporling growth, is provided.

Dried oospore concentrate from Schumacher 2 was chosen for a laboratory direct seeding trial, as this concentrate had the highest germination rate (42 %) determined during the 2003 germination trial.

Schumacher 2's oospore concentrate density was determined. Five mLs of oospore concentrate were weighed, followed by twice-counting the number of oospore in three 0.10 g samples, in order to determine the number of oospores per mL of concentrate. Table 3 (below) illustrates the steps in determining the concentrate density.

Table 3: Determination of Schumacher 2 oospore density per mL from concentrate

	5 ml of oospore wt. (g)			No. oospores counted in 0.10 g			No. oospores per ml		
Trial #	1	2	3	1	2	3	1	2	3
	2.7	2.5	2.4	562 559	502 520	515 520	3024	2555	2481

The oospores counted in trials 2 and 3 (average of 1028) were added to a 40 cm (L) by 20 cm (W) by 25 cm (H) aquarium on April 24, 2003. The sediment comprised of a 2cm thick layer of nutrient-enriched clay layer on the bottom, topped with a 0.5 cm layer of washed masonry sand.

The aquarium was divided into 8 equal quadrants (100 cm ^{2}). The average 1028 oospores were added to an 80 ml water beaker, which was stirred electrically for homogeneity. Ten ml of mixed oospores were extracted with a pipette from the stirring beaker and dispersed evenly to all of the 8 quadrants. Tap water was gently sprinkled up to 7.5 cm inches above the sediment.

Emerging sporling' length were measured and quantified per quadrant, allowing for a percentage estimate of oospore germinated from 1 ml of concentrate.

3.5 Sediment Types and Development

On March 21, 2003 two sediment varying aquaria (32 by 15 cm) were set up.
The first aquarium sediment comprised washed masonry (brick sand) sand, whereas the second aquarium comprised of three sections all containing nutrient enriched clay topped with washed sand and different amendments. Table 4 provides details regarding sediment layering in each aquarium.

Table 4: Sediment Layer Setup *(b, bottom; m, middle; t, top layer)*

	Sediment Type (bottom to top)	Layer Thickness (cm)	Oospores Received	Area (cm2)
Aquarium 1	*Washed masonry sand*	4	250	288
Aquarium 2	*nutrient-enriched clay (b)* *Washed masonry sand (t)*	2 3	160	102
	nutrient-enriched clay (b) *Washed masonry sand (m)* *Bentonite (t)*	3 2 1		78
	nutrient-enriched clay (b) *Washed masonry sand (m)* *Hydrsource (t)*	3 2 1		96

The nutrient-enriched clay was acquired from 30 cm bellow the top sediment of a fertilizer plant retention pond. Out of this clay, a slurry (1:5) in distilled water was prepared for nutrient analysis in May 2003.

Water chemistry (pH, Eh, EC and temperature) was measured for the slurry's supernatant after vigorously stirring for 1 minute and letting it settle for 1 hour. The supernatant was filtered and acidified for elements with nitric acid, and with sulfuric acid for nutrients before being shipped to SRC for analysis.

Phosphorus was determined in-house using the HACH phosphate test kit in combination with the Spectonic 70 spectrophotometer at 890 nm.

Germinated oospores that accumulated from 2003 Trial between March 18 and 21st were transferred equally to the two aquaria. Germinated oospores from March 21st on were transferred into Aquarium #1.

Plastic enclosures for marking specific plants were placed around 3 random *C. vulgaris* sporelings whose length was measured with a ruler four times at one-week intervals for the first month.

4.0 RESULTS

4.1 Oospore Germination by *C. vulgaris*: 2001, 2002 and 2003

Three germination trials were performed using oospores from the five *C. vulgaris* populations; November, 2001 (wet oospores), June, 2002 (dried oospores stored for 96 days) and March 2003 (dried oospores stored for 376 days). Figures 1 to 4 illustrate the % germination in 2001 at 20 ^{o}C (Figure 1) and at 4^{o}C then 20^{o}C

(Figure 3), for 2002 using dried oospores (Figure 4) and for 2003, also using dried oospores (Figure 2) at 20'C.

The laboratory germination trials performed in 2001, 2002 and 2003 testing the same five populations under wet and dry storage conditions resulted in different germination rates.

Figure 1 presents the results of the 2001 wet oospore germination trial. Overall, the highest germination rate, 49 %, was achieved by the St. Clair oospore population. LPL and Sch-2 demonstrated similar percent germination (35% and 31%, respectively). Only 22 % or the Sch-1 oospores germinated, and for Langmuir, only 17 % germinated.

Some variation in the pattern of germination with time can be noted in Figure 1. Virtually all of the St. Clair oospores germinated by day 6, the first observation date. Similarly, most LPL oospores germinated by day 10. Langmuir oospore germination was complete by day 15. However, germination of Sch-1 and Sch-2 oospores was drawn out over more than three weeks during the 2001 wet oospore experiment.

Figure 2 presents the percent germination data for these same five populations following oospore concentrate drying and storage at 4^{o}C for 376 days. Fewer St. Clair oospores germinated in 2003 (30 %) compared to 2001 (49 % (Figure 1). Meanwhile, percent germination of Sch-2 oospores increased in 2003 to 42 %, compared to only 31 % in 2001 (Figure 1).

Percent germination of LPL oospores in 2003 somewhat declined (29 %) compared to 2001 (35 %). 2003 Sch-1 oospore germination was similar to 2001 results. However, percent germination of Langmuir oospores dramatically dropped to only 3 %.

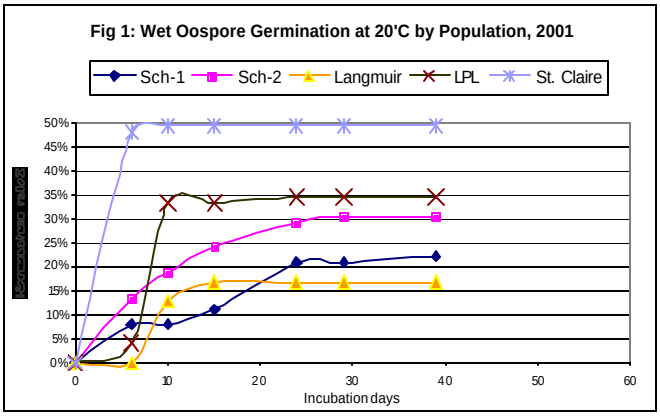
As seen in 2001 (Figure 1), germination of St. Clair and LPL oospores in 2003 was rapid, with most germination complete by day 10. Also, as seen in 2001, Sch-1 oospore germination was drawn out over four weeks.

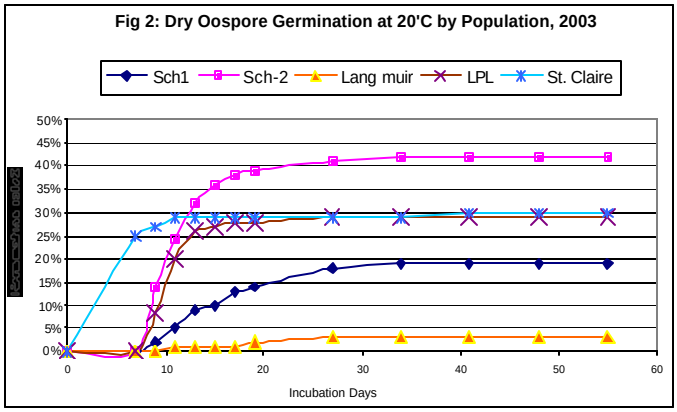
Figure 3 presents the 2001 results of incubating wet oospores of the five populations at 4^{o}C in light In aerobic conditions. No germination occurred during the cold incubation but, upon transfer to 20 ^{o}C, germination of oospores of all five populations was complete within 14 days, with the exception of Sch-2 which, again, demonstrated drawn-out germination over incubation time.

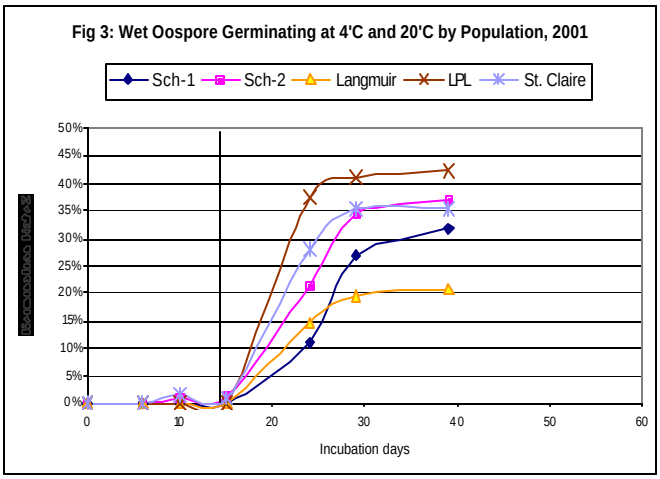
Figure 4 presents the results of the 2002 germination trial using dried oospores stored for 96 days. In this trial, St. Clair oospores had the highest percent germination (35 %), while only 2 % of Langmuir oospores germinated.

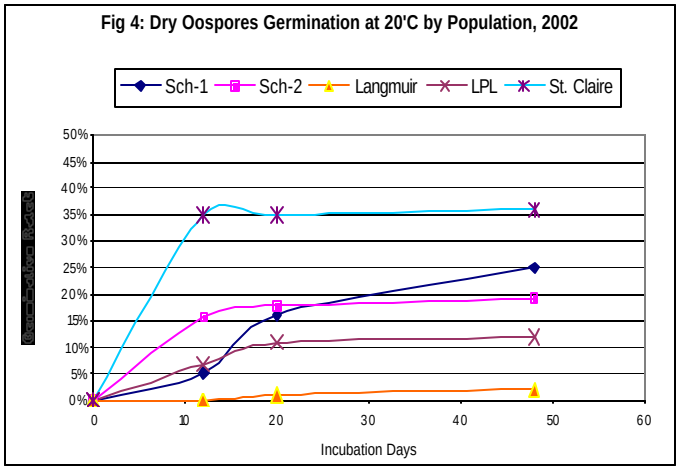
LPL percent germination was low (19 %, compared to both the 2001 wet oospore trial (35 %) and the 2003 dry oospore trial (29 %). This phenomenon was also observed for Sch-2, where the lowest percent germination occurred during the 2002 trial.

Overall, wet St. Clair, LPL and Langmuir oospore populations reached higher average germination rates (49%, 35% and 17%), faster in 2001, whereas dry Sch-2 performed better (42%) in 2003 compared to 31% (wet) in 2001. Sch-1 performed equally both years.









Tables 5a and 5b provide a summary of germination and statistical results for dry vs. wet-stored oospores for the 2001 & 2003 Trials and for the long vs. shorter storage duration; 2002 & 2003 Trials.

Table 5a: % Germination Ranges & Sig. Differences for dry/wet stored oospores at 20C

Population	2001	2003	P<0.05
Storage	**wet (21 d)**	**dry (373 d)**	
St Clair	42% - 56%	28% - 34%	*
LPL	32% - 40%	25% - 37%	*
Sch-1	14% - 28%	16% - 35%	
Sch-2	19% - 46%	39% - 48%	
Langmuir	16% - 18%	3% - 8%	*

Table 5b: % Germination Ranges & Sig. Differences for short/long duration stored oospores at 20C

Population	2003	2002	P<0.05
Storage	**dry (373 d)**	**dry (96 d)**	
St Clair	28% - 34%	34% - 39%	*
LPL	25% - 37%	5% - 18%	*
Sch-1	16% - 35%	19% - 33%	
Sch-2	39% - 48%	10% - 27%	*
Langmuir	3% - 8%	0% - 5%	*

Paired sample T-tests were performed comparing the 2001 wet oospore percent germination with 2003 percent germination of dried oospores (Table 5a). Drying oospore concentrate and storage at 4^{o}C for 96 days significantly reduced percent germination of the St. Clair, LPL and Langmuir oospores. However, percent germination of Sch-1 and Sch-2 oospores was not significantly affected by drying and storage.

The only difference between the 2002 and the 2003 germination trials is the time the dried oospores were stored at 4^{o}C. The results of the paired sample T-tests comparing the 2002 percent germination data with the 2003 data indicate that percent germination significantly increased for LPL, Sch-2 and Langmuir, while the approximately 5 % decline in percent germination of St. Clair dried oospores also registered significant (Table 5b).

Overall, these results indicate that drying and storage of oospores at 4 ^{o}C may reduce percent germination, but this effect appears to be oospore population-specific, given that both Sch-1 and Sch-2 percent germination did not decrease.

The results also indicate that the decreases in percent germination may be reversible upon further storage at 4^{o}C; as described above, increases were noted for three of five populations following cold storage for more than a year.

Water chemistry data for the solutions in Petri plates for 2001 and 2003 are shown in Tables 6 and 7. The redox potential of the Petri dishes with water remained high for both 2001 and 2003; E_7 ranged from 458 to 764 mV after 6 days from set-up and remained high.

The pH of water in the Petri dishes for both years ranged from 7.6 to 8.4, while electrical conductivities ranged from 422 :S.cm^{-1} to 637 :S.cm^{-1} in 2001 trial and 110 :S.cm^{-1} to 390 :S.cm^{-1} in 2003. The water redox potential was always positive; E_7 ranging from 438 to 509 mV in 2001, and 461 to 764 mV in 2003. E_7 values increased after the addition of distilled water to the dishes on April 7th, 2003 (Table 6).

4.2 *C. vulgaris* Establishment from Biomass

Harvesting the transplanted biomass (St. Clair population) after 76 days (23 g wet wt. on April, 16), and re-allowing growth, yielded a 36% lower wet biomass (14.8 g wet wt.) after the second harvest 50 days later (June 3, 2003). However, a separate, first transplant starting out with 11.5 g wet weight ("16-Apr-03 New", Table 8b) yielded a comparable biomass of 11.3 g wet wt. after 30 days (May 15, 2003). Tables 8 a and b summarizes the biomass values together with the water chemistry related to the *Chara* biomass establishment in the 2 tanks.

Table 8 a): Biomass Establishment and Water Chemistry - Tank 1

				Above Sediment Water Chemistry				Surface Water Chemistry			
Date	Process	Wet wt. (g)	Dry wt. (g)	pH	Cond	E_7	T	pH	Cond	E_7	T
30-Jan-03	Transplant	-	-	7.6	323	265	21.1	8.6	322	273	21.1
16-Apr-03	Harvested (1)	23	-	8.0	245	261	20.8	8.8	288	359	21.0
3-Jun-03	Harvested (2)	14.8	3.8	9.7	175	-109	21.5	9.7	170	176	20.7

Table 8 b): Biomass Establishment and Water Chemistry - Tank 2

				Above Sediment Water Chemistry				Surface Water Chemistry			
Date	Process	Wet wt. (g)	Dry wt. (g)	pH	Cond	E_7	T	pH	Cond	E_7	T
16-Apr-03	Transplant	11.5	1.7	7.6	273	369	15.8	7.8	261	325	20.8
15-May-03	Harvested	11.3	1.6	6.6	558	80	21.7	7.6	494	171	21.8

4.3 *Chara* Development on Different Sediment Types

Germinated oospores (all 5 populations) from the 2003 Trial were transferred to Aquarium 1 containing washed sand as the substrate, or to Aquarium 2 with nutrient-enriched clay overlain with sand and bentonite over one-third and Hydrosource over another one third. Sporelings have been measured on a weekly basis for total length development.

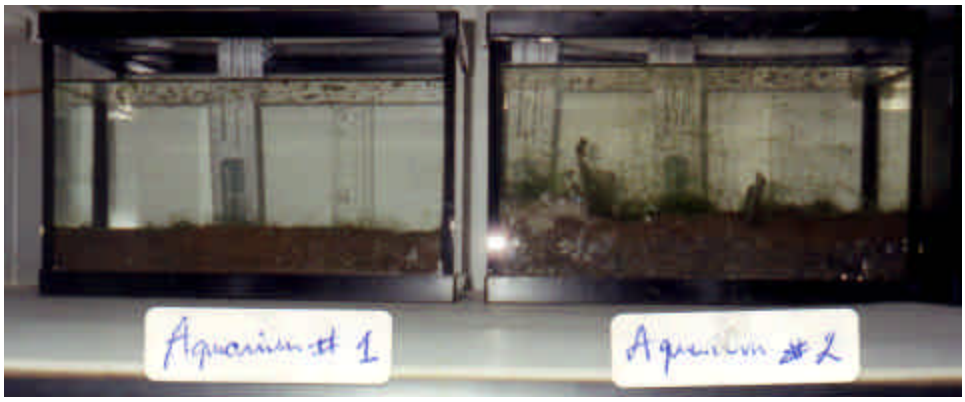
Table 8 below, illustrates the grown length of *C. vulgaris* according to each sediment type. The sediment resulting in the longest shoots was in the aquarium with nutrient-enriched clay overlain with sand and topped with Hydrosource. Sediments containing clay and masonry sand resulted in *Chara* growth averaging of 3 cm per week, while sand alone in Aquarium 1 resulted in 0.1 cm per week. Figure 5 illustrates Aquarium # 1 and 2 before dismantling.

Table 9: *C. vulgaris* Growth Length in Varying Sediment Types

			Days since addition of 1st germ. oospores					Biomass harvested	
			32	39	46	53	60		
Sediment Type (bottom to top)	Thickness (cm)	Enclusure	**Average Sporeling Growth** (cm)					wet wt. (g)	* dry wt. (g)
Washed masonry sand	4	1	1.1	1.2	1.3	1.4	1.6	0.15	0.02
		2	0.9	1.1	1.5	1.6	1.7		
		3	1.2	1.4	1.7	1.7	1.8		
nutrient-enriched clay	2	1	1.8	3.7	6	9.8	16.3	0.8	0.1
Washed masonry sand	3	2	2.2	4	7.1	10.2	16.8		
nutrient-enriched clay	3								
Washed masonry sand	2	1	1.4	3.2	4.3	7.9	13.3	0.7	0.09
Bentonite	1								
nutrient-enriched clay	3								
Washed masonry sand	2	1	1.5	2.9	6.1	16.5	19.8	0.4	0.05
Hydrosource	1								

* dry wt. is estimated on an 86% wet-dry difference established from previous *Chara* experiments

Figure 5: Aquaria #1 and #2 - *Chara* Development 7 Weeks after Setup



The slurry's nutrient content has been determined (SRC Analytical Lab):

Table 10: Nutrient – Enriched Clay Soil Slurry Filtered Supernatant Chemical Composition.

analyte	NH_3	NO_2+NO_3	TKN	TOC	Al	Fe	P *	S	Si	Zn
mg/L	0.89	5	3.9	17	0.12	0.4	0.12	140	1.1	0.4

P* (3.6mg/L) using HACH phosphate test kit at Boojum

4.4 *C. vulgaris* Germination Projection From Oospore Density

Upon introducing 1028 oospores into an 800 cm^2 aquarium, sporelings were observed after 14 days incubation. After 30 days incubation, it is estimated that 10.9% of the oospores originally added had germinated, producing sporelings averaging 3 to 4 cm in length. The number of shoots to date is likely much higher than 113 (10.9%); however due to their density, counting the plants without damaging them would be difficult.

This germination/growth trial demonstrated that about 0.2 grams of Sch-2 oospore concentrate, containing about 1028 oospores (0.54 mL) yielded a minimum of 113 sporelings, or about 1 sporeling per 5 cm^2. These results indicate that, on a suitable seed bed, it may be possible to initiate *Chara* populations using dried oospore concentrates.

5.0 DISCUSSION

5.1 Oospore Germination Trials

The overall results of the 2001, 2002 and 2003 C. vulgaris germination trials indicate that high redox conditions, supplied by tap water in petri plates, induce germination of oospores. In these conditions, percent germination reached as high as 49 % (St. Clair, 2001 wet oospore germination trial).

Following drying and storage of St. Clair oospore concentrate, the percent germination remained quite high, at 35 % in 2002 and 30 % in 2003. However, it was significantly lower than the percent germination prior to drying and storage for this population.

The results indicate that drying and cold storage may significantly reduce percent germination, but not necessarily for every population. Reductions were seen for St. Clair, LPL and Langmuir, while neither Sch-1 nor Sch-2 dried oospores had significantly lower percent germination following dessication and cold storage.

There is some indication that the reductions in percent germination are reversible upon continued cold storage of dried oospores. Significant increases in percent germination were determined for LPL, Sch-2 and Langmuir following long-term cold storage.

High percent germination was not observed for all populations. Only 17 % of Langmuir oospores germinated in the 2001, when wet oospores, directly concentrated from sediment, were used; following Langmuir oospore concentrate drying and storage, percent germination declined to only 2 % in 2002 and 3 % in 2003.

The redox potential of the Petri dishes with water remained high for both 2001 and 2003; E_7 ranged from 458 to 764 mV after 6 days from set-up and remained high.

Dry oospores are likely saturated with oxygen. Upon re-hydration of the oospores, their internal oxygen supply remains despite decreasing dissolved oxygen concentrations in the surrounding solution. Wet oospores stored over time in anoxic sediment may be susceptible to inner O_2 depletion and a lower redox potential if stored for more than a few days/months.

It is possible that, when the dry-stored oospores are incubated in Petri dished with tap water (or at high redox potential), the germination is triggered for a longer period of time as demonstrated in Figure 2 compared to Figure 1. Forsberg (1965) results suggest that air-drying makes oospores become less discriminate in comparison to oospores stored in sediment-like conditions, readily germinating following re-hydration in either high or low redox conditions. Casanova and Brock (1996) also demonstrated high oospore germination from air-dried seed bank sediment samples compared to samples maintained wet during storage.

The difference in response between dry versus wet storage among some populations is less than 15%, thus differences could be attributed to variation among the seed banks rather than real differences in response to storage (e.g. Sch -2 Figures 1, 3 and 4).

The length of storage may also be affecting the oospore germination rate. St. Claire oospore population reached 30% when dried for 14 months and 49% when stored wet for 21 days.

As Casanova and Brock demonstrated in 1996 that, after storing different *Chara* species in dry and wet conditions for 1 month, all oospores germinated quickly, whereas after longer treatment periods, oospore germination was slower and less

synchronous. It may be that oospores germinate quickly when fresh due to more stored resources and have not had time to degenerate.

One obvious source of variation in percent germination between *Chara* seed bank oospore populations is the age/health of the oospores, i.e., populations comprised of younger oospores more readily germinate.

However, it may not be that simple. Alternately, a fraction of oospores grown each year may readily germinate that growing season or the next, leaving viable, but more refractory, oospores to settle in the seed bank. The Schumacher 1 and 2 oospore germination pattern in 2003 clearly demonstrates that some oospores require weeks of sustained aerobic, high redox conditions before they finally germinate. These extremely refractory oospores may represent long term insurance for the population, germinating only after a very long sustained period of high redox.

There is no obvious explanation for why percent germination should decrease for some oospores for some populations, but not others, following desiccation and cold storage.

One possibility is the electrical conductivity of the source water body. For example, significant decreases following desiccation were determined for St. Clair and Langmuir, both low conductivity ponds. During desiccation, salinity of solutions surrounding oospores increases to very high level just prior to complete evaporation of water. Oospores from low conductivity environments may be more sensitive to salinity increases than oospores from high conductivity environments.

A second possibility is that both Schumacher 1 and Schumacher 2 oospores populations were naturally desiccated in the field following a period of drought prior to November 2001, and their overall low percent germination in 2001, combined with no significant decrease in percent germination, may be reflecting this. Drawdown or dry-out of the three other ponds supporting *Chara* populations would be much less likely.

5.2 Review of Related Literature

The ecological importance of suspended solids control by Characeae had been recognized (Simons et al.,1994),and the relegating of ^{226}Ra to the sediment has been documented (Kalin, 2001).

A recent study conducted in Switzerland demonstrates that less frequent Characeae species such as *C. aspera*, *C. major* and *N. syncarpa* are being replaced by more frequent species: *C. globularis* and *C. vulgaris* at the level of the whole country, as well as in Netherlands (Simons and Nat, 1996; Joye A. et al. 2002). It has been suggested that this species replacement is a consequence of eutrophication or nutrient enrichment of sediments and water.

Among submerged macrophytes, Charophytes have been documented as fast colonizers in temporal or disturbed habitats (Wade, 1990) mostly due to the production of high numbers of small oospores. Oospore densities commonly reach 10^6 per m^2 in the top 5 to 10 cm of sediment (Casanova& Brock, 1999; Smith, in preparation). Proctor (1967) found that oospores are very persistent, therefore ensuring oospore banks that could last for decades. In the long run, buried oospores could ensure the survival of charophyte populations during the period when conditions are unfavorable for their growth.

Germination of less than 10%, even after prolonged stratification is common, due to dormancy exhibited after oospores are released from the plant, according to Proctor (1967). However, according to a study (Bonis and Lepart , 1990), germination of *C. aspera* and *C. canascens* germination in the seed bank sediment was limited to the top 2cm (22 to 48% germination), while germination below this stratum was less than 1%.

With increasing burial depth, germination might decrease because of attenuation of germination-inducing factors, e.g. temperature fluctuations or exposure to light (Bones and Lepart, 1994). Furthermore, the availability of oxygen in high E_7 to oospores at or near the sediment surface may be the specific trigger for germination.

Spatial and temporal variation in the distribution of Charophytes in waters suggest that the requirements for breaking dormancy and germination varies among species (Casanova and Brock, 1990). *In vitro* experiments dealing with specific environmental conditions supporting *Chara* oospore germination have been examined (Proctor, 1967; Casanova and Brock, 1996). Suggested environmental variables affecting oospore germination include: thermal stratification, desiccation, seasonal germination windows, chemical micro-environment (redox potential and dissolved oxygen), and pre-dehiscence growth inhibitors. Germination in the dark has been reported for some species: *Chara gymnopitys* (Carr and Ross, 1963), *Chara* cf. *aspera* reported by Bonis and Grillas, 2002 (van den Berg, 1999) and *C. vulgaris* (Smith, in preparation).

Proctor (1967) examined storage conditions and concluded that storage settings affect germination rates. *Chara* oospore germination stored as wet concentrates at 3^{o}C decreased over 4 years of storage, while air-dried oospores retained their viability when germinated in autoclaved soil and tap water.

High *in vitro* germination of *C. australis* (41%) occurred after storing the oospores in wet conditions for 4 months, whereas greatest germination for *C. muelleri* was achieved after dry storage in either dark or ambient light conditions (Casanova and Brock, 1996).

5.3 *Chara* Laboratory Growth Rate Determination

Chara growth rates were determined in the lab in small aquaria under artificial lighting at constant room temperature. Growth rates ranging from 0.71 to 0.58 gdw.m^{-2}.d^{-1} were determined in the lab

Growth of *C. vulgaris* has been strongly related to the sediment type. The key factor has been the clay layer - rich in NO_2+NO_3 (5.0mg/L), P (3.6mg/L) and S (140 mg/L) with a high conductivity of 960 $:S.cm^{-1}$.

Biomass transplants, providing the respective conditions, have been successful for *Chara* establishment. Our wet biomass weights of established *Chara* from transplants demonstrate a 90% biomass return. However this holds true only for the first time transplant, as the transplanted bunch contains numerous intertwined habits or axillary fertile heads from which new shoots rise, and have a higher nutrient recycling capacity. After harvesting, growth from the sediment takes place, where nutrients have been depleted, thus reducing the yielded biomass.

5.4 Optimizing Substrate Configurations For Germination and Sporeling Development

The germination test methodology includes tap water in Petri plates. This medium's high redox condition can be maintained in this configuration. However, the results of these and earlier growth trials have demonstrated the necessity of fine grained sediments such as clay for rapid development of *Chara* sporelings. These materials offer very large surface areas and harbor dense population of heterotrophic bacteria which consume oxygen, lowering the redox.

The oospore germination trial performed in the aquarium containing clay overlain with a thin layer of sand provided suitable conditions for both oospore germination and sporling growth. Practically, placement of clay over engineered pond bottoms will be relatively simple. However, placement of a consistent 1 cm thick layer of washed sand over this clay will be a technical challenge in the field.

Table 6: Water Germination Conditions, 2003

Room Temp		pH	Em	T C	Cond	E7
Sch-1	17-Mar-03	8.0	221	21.7	178	520
	20-Mar-03	8.2	256	20.7	111	568
	22-Mar-03	8.2	295	20.3	111	611
	24-Mar-03	8.1	242	20.5	112	548
	26-Mar-03	8.3	250	19.1	115	568
	28-Mar-03	8.3	213	18.7	123	534
	30-Mar-03	8.4	190	19.5	174	515
	7-Apr-03	8.1	218	15.9	215	531
	1-May-03	8.0	307	20.7	132	606
	6-May-03	7.9	440	20.5	167	738
Langmuir	17-Mar-03	7.7	251	22.4	291	535
	20-Mar-03	8.1	260	20.3	115	566
	22-Mar-03	8.1	290	20.7	111	600
	24-Mar-03	8.0	263	20.7	116	565
	26-Mar-03	8.1	257	19.6	115	567
	28-Mar-03	8.2	249	19.2	121	566
	30-Mar-03	8.3	229	19.7	115	548
	7-Apr-03	8.2	211	16.2	134	530
	1-May-03	7.7	275	21.1	351	561
	6-May-03	7.6	420	20.4	390	697
St. Claire	17-Mar-03	7.8	250	22.6	105	536
	20-Mar-03	8.1	270	20.9	117	580
	22-Mar-03	8.1	276	20.9	111	585
	24-Mar-03	8.1	234	19.7	110	545
	26-Mar-03	8.2	250	19.5	115	564
	28-Mar-03	8.3	239	19.3	114	559
	30-Mar-03	8.4	194	20.1	116	520
	7-Apr-03	8.3	197	16.1	342	520
	1-May-03	8.0	285	20.9	335	588
	6-May-03	8.0	232	20.7	136	535
Sch-2	17-Mar-03	7.6	258	22.7	156	537
	20-Mar-03	8.1	255	20.7	114	562
	22-Mar-03	8.0	268	20.8	108	569
	24-Mar-03	8.2	265	19.1	111	580
	26-Mar-03	8.2	253	19.7	110	568
	28-Mar-03	8.2	238	19.3	112	554
	30-Mar-03	8.2	213	20.0	113	528
	7-Apr-03	7.4	191	16.0	390	461
	1-May-03	7.7	280	21.6	173	562
	6-May-03	7.9	465	19.5	280	764
LPL	17-Mar-03	7.9	216	22.9	110	512
	20-Mar-03	8.2	263	20.8	114	576
	22-Mar-03	8.2	300	20.2	111	615
	24-Mar-03	8.3	271	19.3	123	589
	26-Mar-03	8.1	273	19.8	124	581
	28-Mar-03	8.1	222	19.1	134	531
	30-Mar-03	8.3	212	19.7	126	533
	7-Apr-03	8.2	170	15.5	440	484
	1-May-03	8.0	277	20.9	142	581
	6-May-03	7.8	369	19.6	152	662
Tap water	17-Mar-03	7.9	247	22.5	286	540
	20-Mar-03	7.9	286	19.9	171	584
	22-Mar-03	8.3	295	19.7	120	615
	24-Mar-03	8.1	236	20.5	132	543
	26-Mar-03	8.3	256	19.0	136	578
	28-Mar-03	8.5	205	19.0	155	536
	30-Mar-03	8.4	190	19.8	143	516
	7-Apr-03	8.4	196	16.2	470	524
	1-May-03	7.4	257	21.8	278	525
	6-May-03	7.8	450	19.5	307	742

Table 7: Water Germination Conditions, 2001

Room Temp		pH	Em	T C	Cond	E7	pH	Em	T C	Cond	E7	pH	Em	T C	Cond	E7
Population A	26-Nov-01															
	2-Jan-02	8.17	148	19.3	613	461	8.17	146	19.50	561	458	8.22	150	19.2	555	466
Population B	26-Nov-01															
	2-Jan-02	8.2	148	19.3	594	462	8.21	149	19.30	610	464	8.21	145	19.7	637	460
Population C	26-Nov-01															
	2-Jan-02	8.21	185	19.0	584	500	8.43	181	18.20	569	509	8.30	179	19.6	630	499
Population D	26-Nov-01															
	2-Jan-02	8.35	174	19.7	632	497	8.33	168	20.00	669	489	8.32	169	19.9	601	490
Population F	26-Nov-01															
	2-Jan-02	8.14	161	19.4	613	472	8.19	164	19.50	527	478	8.17	159	19.5	570	471
4 ^{o} C (Fridge)																
Population A	26-Nov-01															
	2-Jan-02	7.92	159	19.8	555	457	8.04	163	19.00	608	468	8.08	151	19.5	422	458
Population B	26-Nov-01															
	2-Jan-02	8.12	148	18.9	506	458	8.10	141	18.60	468	450	7.97	137	19.7	477	438
Population C	26-Nov-01															
	2-Jan-02	7.95	133	19.0	656	433	8.11	137	19.00	543	446	8.12	138	19.3	527	448
Population D	5-Dec-01															
	2-Jan-02	8.13	134	19.3	491	444	8.18	144	19.30	543	457	8.05	154	18.6	545	460
Population F	19-Dec-01															
	2-Jan-02	8.11	148	19.2	542	457	8.19	143	19.30	540	457	8.18	146	19.0	548	459

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7.0 APPENDIX

Paired Sample T -test (Accumulative Germ) Wet - Dry

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	LPL01	28.0000	15	13.18007	3.40308
	LPL03	21.8000	15	13.34809	3.44646
Pair 2	SCH101	14.0000	15	7.34847	1.89737
	SCH103	15.0667	15	11.35446	2.93171
Pair 3	STCLAI01	48.4000	15	5.40899	1.39659
	STCALI03	30.6000	15	2.64035	.68173
Pair 4	SCH201	23.4000	15	10.17560	2.62733
	SCH203	26.3333	15	16.34305	4.21976
Pair 5	LANGM01	12.5333	15	6.73866	1.73991
	LANGM03	2.4667	15	2.23180	.57625

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	LPL01 & LPL03	15	.830	.000
Pair 2	SCH101 & SCH103	15	.410	.129
Pair 3	STCLAI01 & STCALI03	15	-.428	.111
Pair 4	SCH201 & SCH203	15	.546	.035
Pair 5	LANGM01 & LANGM03	15	.609	.016

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LPL01 - LPL03	6.2000	7.73859	1.99809	1.9145	10.4855	3.103	14	.008
Pair 2	SCH101 - SCH103	-1.0667	10.70024	2.76279	-6.9923	4.8589	-.386	14	.705
Pair 3	STCLAI01 - STCALI03	17.8000	6.96112	1.79735	13.9451	21.6549	9.903	14	.000
Pair 4	SCH201 - SCH203	-2.9333	13.75015	3.55027	-10.5479	4.6812	-.826	14	.423
Pair 5	LANGM01 - LANGM03	10.0667	5.66274	1.46211	6.9307	13.2026	6.885	14	.000

Paired Sample T-tests (Accumulative Germ.) Dry - Dry

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	LPL02	9.9333	15	6.12334	1.58104
	LPL03	27.8000	15	4.66292	1.20396
Pair 2	SCH102	15.7333	15	9.38438	2.42304
	SCH103	17.8667	15	9.63525	2.48781
Pair 3	STCLAI02	35.3333	15	2.28869	.59094
	STCALI03	30.1333	15	2.55976	.66093
Pair 4	SCH202	17.8000	15	6.72097	1.73535
	SCH203	35.2667	15	8.37058	2.16128
Pair 5	LANGM02	.9333	15	1.57963	.40786
	LANGM03	2.6667	15	2.05866	.53154

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	LPL02 & LPL03	15	-.011	.970
Pair 2	SCH102 & SCH103	15	.848	.000
Pair 3	STCLAI02 & STCALI03	15	.736	.002
Pair 4	SCH202 & SCH203	15	.037	.897
Pair 5	LANGM02 & LANGM03	15	.124	.659

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	LPL02 - LPL03	-17.8667	7.73551	1.99730	-22.1504	-13.5829	-8.945	14	.000
Pair 2	SCH102 - SCH103	-2.1333	5.24904	1.35530	-5.0402	.7735	-1.574	14	.138
Pair 3	STCLAI02 - STCALI03	5.2000	1.78085	.45981	4.2138	6.1862	11.309	14	.000
Pair 4	SCH202 - SCH203	-17.4667	10.54153	2.72181	-23.3044	-11.6290	-6.417	14	.000
Pair 5	LANGM02 - LANGM03	-1.7333	2.43389	.62843	-3.0812	-.3855	-2.758	14	.015