Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland

Paul J. Oberholster • Christian Blaise • A.-M. Botha

P.J. Oberholster

CSIR Natural Resources and the Environment, P.O. Box 395, Pretoria 0001, South Africa

e-mail: poberholster@csir.co.za

C. Blaise

Science and Technology Branch, Environment Canada, St Lawrence Centre, Montréal QC H2Y 2E7, Canada

A.-M. Botha

Department of Genetics, University of Stellenbosch, Private Bag X1, Matieland, Stellenbosch, 7601, South Africa.

Abstract The occurrence of a sunflower oil spill in 2007 in the Con Joubert Bird Sanctuary freshwater wetland, South Africa, inhibited the growth of sensitive phytoplankton species and promoted that of tolerant species. The algal divisions Chlorophyta and Euglenophyta were well represented in the sunflower oil contaminated water, especially the species *Euglena sociabilis*, *Phacus pleuronectes* and *Chlamydomonas africana.* Young and mature resting zygotes of *Chlamydomonas africana* were recorded in high abundance at all the sunflower oil contaminated sampling sites. The phytobenthos diversity and abundance were significantly suppressed and negatively associated with low Dissolved Oxygen concentrations and the negative redox potential of the bottom sediment. At the intracellular level, phytoplankton chlorophyll *a* and *b* concentrations as physiological variables were more sensitive indicators of the adverse effects of sunflower oil than the 72 hour *Selenastrum capricornutum* algal bioassay conducted.

Keywords *Chlamydomonas africana,* cyanobacteria, chlorophyll *a* and *b* concentrations, light intensity, algal bioassay

Introduction

When a petroleum or non-petroleum oil spill occurs, adverse effects on the surrounding ecosystem can occur as a result of exposure. Wetland ecosystems especially are vulnerable due to the fact that they are close ecosystems which provide critical feeding, spawning, and nursery habitats for numerous species (Mitsch and Gosselink 1993). With a high human population growth and its consequent demands

62 on limited water resources, more than one-third of South Africa's wetlands have been
63 destroyed (Breen and Begg, 1989). Those that still remain are increasingly threatened
64 by pollution (Begg, 1990). Spilled oil in wetland areas can be transformed through a
65 wide range of physical, chemical, and biological weathering processes that changes
66 the composition, behaviour, exposure routes, and toxicity of the oil (USDOC/NOAA
67 1996). Whether the environmental fate and toxicity of the transformed products differ
68 from that of the parent depends upon the specific oil and product that were formed.
69 Generally, vegetable oils and petroleum oils are of low viscosity and the spread of
70 these oils over a large area will hamper its recovery (Groenewold 1982). Since
71 vegetable oils and animal fats usually have few volatile fractions, and therefore
72 usually do not decrease in volume through evaporation as do many of the lighter
73 factions of petroleum oils, most of the quantity of spilled vegetable oil and animal fats
74 remain in the environment (Rigger 1997). When this happens, there is a potential for
75 adverse impacts to environmentally sensitive areas. Factors that affect the
76 biodegradation of oils include pH, dispersal of oil, dissolved oxygen, occurrences of
77 nutrients in the proper proportions, soil types, type of oil, and the concentration of
78 undissociated fatty acids in the water (Cornish et al. 1993, Rigger 1997).

79 In addition, vegetable oils and animal fats may biodegrade more quickly than
80 petroleum. However, in the short term, this advantage is neutralized by the ability of
81 many petroleum compounds to evaporate quickly (Groenewold 1982). Hence, both
82 kinds of oil will degrade more slowly in low-energy waters (stagnant waters with little
83 movement) and can become submerged in an anoxic aquatic habitat, settle to the
84 bottom and into sediments, or form thick layers (Groenewold 1982).

85 Although vegetable oil spills have been found to be deleterious to different
86 organisms, their impact on phytoplankton communities in a freshwater wetland

87 environment have not been previously studied. Furthermore, although vegetable oils
88 lack the acutely volatile components that are present in petroleum and its refined
89 products (e.g. aromatic hydrocarbons), it can still cause severe damage to sensitive
90 aquatic organisms and ecosystems (Mudge 1995).

91 Field and laboratory studies on the effects of vegetable oil on marine, river and
92 coastal marsh environments are numerous (e.g. Zoun et al. 1991, Mudge et al. 1993,
93 Mudge 1995). However, our ability to predict the effects of a vegetable oil spill on
94 freshwater wetlands and especially phytoplankton communities is limited due to the
95 fact that only a few studies address the many factors controlling the response of
96 freshwater wetland ecosystems to oil contamination (Oberholster et al. 2008).
97 Laboratory studies generally allow for a detailed study of one to several parameters of
98 vegetable oil, under a relatively narrow range of controlled conditions, and therefore
99 provide limited application to freshwater wetlands where numerous environmental
100 factors may play a role on phytoplankton assemblage (Mitsch and Gosselink 1993).
101 Again, data gathered from a vegetable oil spill in a freshwater wetland and its possible
102 adverse effects on phytoplankton communities can be difficult to interpret because of
103 the lack of pre-spill site characterization and difficulties in establishing post-spill
104 control or reference sites. Furthermore, several important questions regarding the
105 mechanisms of vegetable oil actions at the cellular and metabolic level remain
106 unanswered (Crump-Wiesner and Jennings 1975, Mudge 1995, Oberholster et al.
107 2008). The objectives of this study were (1) to compare post-spill physical and
108 chemical variables at different contaminated sampling sites with phytoplankton
109 abundance and diversity, (2) to use an algal bioassay to determine growth inhibition
110 or stimulation of *Selenastrum capricornutum* exposed to undiluted sunflower oil
111 contaminated water of the different sampling sites and, (3) to use chlorophyll a and b

112 concentrations as a physiological index of possible changes that may have occurred at
113 the cellular level in phytoplankton exposed to sunflower oil contaminated water.
114
115 **Materials and methods**
116
117 Study area
118
119 The study was conducted in January 2008 on the Con Joubert Bird Sanctuary wetland
120 (26° 11' 20" S 27° 41' 03"E), which acts as a habitat for 230 bird species. The 12.7-ha
121 freshwater wetland has a maximum depth of 1.2 m (in the rain season) and its
122 marginal vegetation was dominated by *Phragmites australis* and *Typha capensis* (Fig.
123 1, Table 1.). In the beginning of September 2007, a spill of 250 ton sunflower oil
124 occurred at a vegetable oil storage facility in Randfontein, South Africa, when a
125 sunflower oil storage tank collapsed. The vegetable oil spilled inside the facility and,
126 due to the volume of oil, the multiple trapping systems were overloaded and some of
127 the oil followed the storm water drains into the Con Joubert Bird Sanctuary wetland
128 area. To the authors' knowledge this oil spill was the largest of sunflower oil in a
129 freshwater wetland environment in the world.
130 The wetland is a transitional open freshwater wetland type with an open water
131 zone (Morant 1983). The water budget of the Con Joubert Bird Sanctuary wetland
132 area is governed by evaporation, precipitation and the inflow of storm water inlets,
133 making the wetland a low-energy budget aquatic system with reduced flushing
134 especially in the dry season months (April-August). The immediate surrounding land-
135 uses of the wetland are industries and urban development. Mechanical techniques
136 were employed as cleanup measure after the sunflower oil spill occurred in 2007.

137 Inflatable booms were used to isolate the contaminated area of marginal vegetation
138 from the open water zone to prevent further contamination, while free oil
139 accumulating between reeds and vegetation on the western side of the wetland was
140 collected using absorbent material and inflatable booms. Large quantities of oil (175
141 tons) were recovered from the surface water by means of a Rotodisc skimmer.

142 The study was conducted 30 days after mechanical clean-up activities of the
143 oil spill were completed and in the time frame period before biostimulation activities
144 of natural microbial populations commenced in 2008. Because of the lack of pre-spill
145 site characterization and difficulties in establishing post-spill control or reference
146 sites, phytoplankton abundance and diversity were compared between different
147 sampling sites with varying amounts of sunflower oil within the water column and
148 bottom sediment, as well as chemical/physical parameters e.g. pH and
149 biological/biochemical oxygen demand (BOD). As a result of the short (two month)
150 period of time after mechanical clean-up activities were finished and before
151 biostimulation of natural microbial communities with fertilizer started, our two
152 weekly sampling frequencies in January and February 2008 were confined to
153 phytoplankton abundance and diversity and did not include a seasonal successional
154 sequence of phytoplankton assemblage. Water samples were analysed within one
155 week after collection. The six permanent sampling sites were selected on the basis of
156 excesebility for sampling between the reedbeds of the wetland as well as with a
157 substratum that consisted predominantly of clay. All permanent sampling sites were
158 sampled on a two weekly intervals.

159

160 **Physicochemical measurements of oil contamination in the water column and**
161 **sedimen**t

162

163 A sidering sampler modified from the design of Baker et al. (1985) was used to
164 sample sunflower oil concentrations in the water column at 0.25 metre intervals from
165 the surface down to the bottom. At each interval of 0.25 metre, 250 ml of water
166 column water was sampled with the sidering sampler. A random sampling procedure
167 was used for the three replicate samples that were sampled at each of the 6 permanent
168 sampling sites to reduce hydrobiological variability and possible movement of
169 sunflower in the water column. Each of the three replicated water column (0.25 metre
170 intervals) samples were combined to form three single composite samples for each of
171 the 6 sampling site. These three single composite samples at each permanent sampling
172 site were then combined to form a single representitive sample (4 litres) for each of
173 the 6 selected permanent sampling sites. The singel representitive sample for each site
174 was used in this study, since water column depth between the 6 sites varied during
175 our study period of 2 months (Table 4). For measurements of sunflower oil within the
176 water column, phytoplanton identification, general water chemistry and algal
177 bioassays, sub-samples of 1 litre each of the single representitive sample (4 litres) of
178 the 6 selected sampling sites were used. Water column samples for biochemical
179 oxygen demand was taken seperatly at each site during each sampling site visit. All
180 samples were kept in coolers with ice packs during the 1-h period of transfer from the
181 field to the laboratory. To prevent cross-contamination of sunflower oil in the water
182 column of different sampling sites, the syringe sampler was decontaminated between
183 each sampling site using hexane and acetone. Bottom sediment sampling was
184 conducted with a Perspex sediment corer (5 cm in diameter) down to a sediment depth
185 of 5 cm, to investigate the spatial extent of sediment oil contamination (Oberholster et
186 al. 2006) while 250 g sediment was also collected at each of the sampling sites, dried

187 to constant weight (105 ^{o}C), cooled and sieved to obtain particle size. Organic matter
188 content of these samples were determined gravimetrically from 50 g test portions of
189 unsieved material after ashing at 500 ^{o}C for 8 h. Dissolved inorganic nitrogen (DIN),
190 soluble reactive phosphorus (SRP) and sulphur were analyzed using classical
191 spectophotometric methods (American Public Health Association, American Water
192 Work Association, and Water Pollution Control Federation 1980). Sunflower oil in
193 the sediment and the water column were determined by using the US Environmental
194 Protection Agency (EPA) Gravimetric method 413.1 (Code of Federal Regulations,
195 Part 136, 1994). Temperature profiles, pH, Dissolved Oxygen (DO) and conductivity
196 of the water column were measured at the surface and at a depth of 0.5 metre with a
197 Hach^{TM} sension 156 portable multiparameter (Loveland, CO, USA). Transparency
198 (Z_{SD}) in the open water zone was measured with a 20 cm diameter, black and white
199 quadrant Secchi disk.
200

201 **pH and redox potential (Eh) of sediment**

202

203 pH and Eh measurements were taken on site from sediment cores of all sampling
204 sites. Measurements were taken from the surface sediment layer and at a depth of 5
205 cm. The pH of the first cm of sediment was measured with a glass combination
206 electrode (AGB-51) and the Eh was measured using a platinum electrode (AGB-51).
207

208 **Determination of biochemical oxygen demand**

209

210 Sampling for biological/biochemical oxygen demand (BOD) was done seperatly
211 during each of the 4 field visits at the selected 6 sampling sites. Grab samples of

212 surface water were put in 2-litre plastic bottles and held on ice in insulated cooler
213 boxes for transport to the laboratory. The maximum time between sample collection
214 and initiation of analysis was 3 hours. In the laboratory two 300 ml BOD bottles for
215 each site were filled to overflowing with the collected water from each sampling site
216 after temperature, pH and DO were adjusted. DO concentration of each bottle was
217 measured, and bottles were stoppered, capped and incubated. Primary standard
218 solution for BOD were prepared from 1:1 mixtures of glucose and glutamic acid
219 (Clesceri et al., 1998). BOD was determined using the standard method of Hauer and
220 Lamberti (2006), by incubating the samples at 20 ºC in the dark for 5 days. After the
221 first 24 h, DO concentrations in the BOD bottles were recorded and the samples were
222 aerated by an oil-free aquarium air pomp until the DO concentrations was above 8 mg
223 l^{-1}. Dissolved oxygen was measured again after 48 h of incubation for those samples
224 that contained less than 4 mg l^{-1} DO after 24 h of incubation. After 72 h of incubation,
225 DO was measured and all samples were re-aerated. The samples with less than 4 mg
226 l^{-1} DO after 72 h incubation was measured again at 96 h. The final DO measurement
227 was made after 120 h. The BOD concentration was calculated by summing the losses
228 of DO during the 5 day incubation.
229
230 **Phytobenthos and phytoplankton sampling**
231
232 Three random samples (100 ml each) for phytobenthos identification were sampled
233 during the four sampling trips at each of the permanent sampling site using a Willner
234 sampler and stored in a cool box in the dark until preparation in the laboratory
235 (Oberholster et al. 2005). These samples were fixed with buffered 5 % (v/v)
236 formaldehyde in the field for determination of phytobenthos composition, community

237 structure and identification of species present. A total of 50 ml of each of the samples
238 were sedimented in chambers and were analyzed under an inverted microscope at
239 1250 x magnification using the strip-count method (American Public Health
240 Association 1989). Diatoms were identified after clearing in acid persulfate. The
241 biovolumes of the more abundant taxa were estimated by measuring cell dimensions
242 of at least 20 individuals and using the closest geometric formulae (Willen 1976). A
243 sub-sample (1 litre) of the single representitive sample (4 litres) for each of the 6
244 selected sampling sites were used to determine phytoplankton assemblage within the
245 water column. The sub-sample were preserved in the field by addition of buffered 5
246 % (v/v) formaldehyde. Phytoplankton identifications were made according to Wehr
247 and Sheath (2003), Van Vuuren et al. (2006) and Taylor et al. (2007). The total
248 number of phytoplankton taxa and their frequency of occurrence at each sampling site
249 were categorised according to Hörnström (1999): $1 \leq 250$, 2 = 251-1000, 3 = 1001-
250 5000, 4 = 5001-25 000 cells l^{-1}. Strip counts were made until at least 300 individuals
251 of each of the dominant phytoplankton species were counted. Phytoplankton diversity
252 was calculated using Shannon's diversity index (Shannon and Weaver 1949).
253
254 ***Selenastrum capricornutum* bioassay and chlorophyll concentrations**
255
256 *S. capricornutum* (syn. *Raphidocelis subcapitata*) which is a non-motile, unicellular,
257 crescent-shaped, green alga, 40 to 60 μm in size was used as test species in the
258 bioassay conducted on the undiluted water of the 6 sampling sites. It is free of
259 complex structures and, therefore, does not clump or form chains. This alga is
260 reported to be generally sensitive to a wide array of (in)organic contaminants and is
261 extensively used in local and international standard toxicity tests (Slabbert 2004). The

test culture used in this study was originally obtained from the St. Lawrence Centre, Environment Canada, as ATCC 22662. Algal culturing and growth inhibition testing were carried out according to standard procedures (Slabbert 2004).

To prevent any possible adverse effects of low DO to the test algae, due to the presence of high concentrations of sunflower oil in the sampled water, two batches of 72 hour algal bioassays were conducted with undiluted wetland water of the different sampling sites. The first batch of un-aerated water samples was tested in sterile 24-well microplates, while a second batch of aerated water samples (20 air bubbles/minute) were carried out in 5 replicate sterile glass test tubes containing undiluted wetland water of each of the 6 sampling site, including triplicate controls containing containing Milli-Q® water and AAP meduim had the following chemical composition, per litre: 25 mg $CaCl_2.2H_2O$; 0.78 μg $CoCl_2.6H_2O$; 0.009 μg $CuCL_2.2H_2O$; 12.16 mg $MgCl_2.6H_2O$; 96 μg $FeCL_3.6H_2O$; 185.64 μg H_3BO_3; 175 mg K_2HPO_4; 75 mg $MgSO_4$; 264.27 μg $MnCL_2.4H_2O$; 7.26 μg $Na_2MoO_4.2H_2O$; 15 mg $NaHCO_3$; 250 mg $NaNO_3$; 32.7 μg $ZnCL_2$; and 333 μg $Na_2EDTA.2H_2O$. Samples were inoculated with 4-day old logarithmic growth phase cells at a density of 1×10^5 cells/ml (Ross et al. 1988). After 72 h of incubation, growth was determined via optical density (OD) using a microplate reader (450 nm). The effect on algal growth was determined as percentage inhibition or stimulation. In the test an inhibition of ≥ 20 % over controls indicates toxic activity, while growth ≥ 20 % over controls indicates stimulation. The following water quality parameters: pH, alkalinity, hardness and temperature, were measured at the start and end of each bioassay test. A third and fourth batch of un-aerated algal bioassays were also conducted with non-sterile (water samples directly from wetland) and sterile water (water that was filtered through a 0.25 μm Whatman filter before use in the algal bioassays) to assess the

287 possible influence of other phytoplankton taxa and micro-organisms in the test water
288 which could confound test results. The latter batch of bioassays were conducted over
289 a period of 96 hours in 5 replicate glass test tubes for each sampling site, plus
290 triplicate controls to determine chronical effects of sunflower oil. For the
291 determination of chlorophyll (chl) *a* and *b*, 1 ml aliquots of the algal suspensions in
292 the test tubes containing sterile wetland water of the fourth batch of bioassays were
293 removed at 24 h intervals over a period of 96 h. Chl was extracted into 80 % acetone
294 at 4ºC. Chl *a* and *b* concentrations were determined spectrophotometrically (647 nm
295 and 664 nm wavelengths) according to the method of Porra et al. (1989).

296

297 **Data analyses**

298

299 In the statistical analysis the Pearson's correlation coefficient, Turkey test and
300 Canonical Correlation Analysis (CCorA) were used. Statistical significance of factors
301 (CCorA) was tested by χ^2 test. All computation was done using MVSP 3.11, Statistica
302 5.0 and XLSTAT Version 2009.6.04. The statistical analysis of the CCorA comprised
303 of the relationship between the biomass of phytoplankton species and environmental
304 variables. Calculations were done for six species: *Chlamydomonas africana*,
305 *Oscillatoria princeps*, *Anabaena flos-aquae*, *Fragilaria ulna*, *Fragilaria capucina*
306 and *Navicula viridula*.

307

308 **Results**

309

310 **Phytoplankton species diversity and abundance**

311

312 In this study very low phytobenthos and phytoplankton species diversity was recorded
313 at sampling sites 1, 2, 3, 4 and 6 ,while sampling site 5 had the highest diversity (*H′* =
314 2.83). However, the average high phytoplankton numbers (1001-5000 cells l^{-1}) of the
315 algal divisions Chlorophyta and Euglenophyta namely *Euglena sociabilis*, *Phacus*
316 *pleuronectes*, *Chlamydomonas africana* and young and mature resting zygotes of
317 *Chlamydomonas africana* were recorded at all 6 sampling sites (Table 2). Site 2 had
318 the lowest phytoplankton species diversity (*H′* = 1.62) indicating that the highest
319 possible impact of oil on the phytoplankton community may have occurred at this site.
320 Phytobenthos abundance (≤ 200 cells per cm^2) was sharply suppressed at sampling
321 sites 1, 2, 3, 4 and 6 with the lowest species diversity at sites 1 and 2 (*H′* = 1.91; 1.62).
322 We observed a significant relationship between the low benthic diatom species
323 (excluding *Fragilaria ulna*) abundance (≤ 200 cells per cm^2) at sampling sites 1, 2, 3,
324 4, 6 ($p \leq 0.05$; $r = 0.956$) and the low DO (1.3, 1.6, 2, 2.1 and 2.5 mg l^{-1})
325 concentrations and the negative redox potential (-190, -187, -225, -211 and -209 mV)
326 that exist within the first 5 cm of the sediment of these sites (Tables 2 and 3). The
327 filamentous cyanobacteria *Oscillatoria princeps* was the dominant cyanobacterial
328 species in the water column of sites 3, 4 and 6 with the highest biovolume of this
329 species (15 mm^3 l^{-1}, 1001-5000 cells l^{-1}) observed at site 3, which was also the
330 sampling site containing the highest sunflower oil concentration (81.5 mg l^{-1}) in its
331 water column (Tables 3 and 4). The high biovolume of the species correlated
332 positively with the occurrence of high oil in the water column ($p < 0.05$; $r = 0.968$)
333 (Tables 3 and 4, Fig 4). However, at sampling site 5 the filamentous cyanobacteria
334 *Anabaena flos-aquae* with a much higher biovolume of 12 mm^3 l^{-1} (1001-5000 cells l^{-1})
335 in comparison with the other sampling sites showed a significant inverse correlation

336 ($p < 0.05$; $r = -0.819$) with the low average total nitrogen concentration (0.037 mg l^{-1})
337 measured at this site (Table 4, Fig 4).

338 The BOD levels (144 mg l^{-1}) were much higher in the water column of site 5
339 in comparison to sites 1, 2, 3 and 6 where BOD concentrations were lower (24, 24, 30
340 and 44 mg l^{-1}) (Table 3). *Fragilaria ulna* was the only diatom species that occurred at
341 5 out of the 6 sampling sites, indicating that this species was more tolerant to the
342 adverse effects of vegetable oil than the other diatom species identified throughout the
343 study. From the diagram it resulted out that *Fragilaria ulna* appeared independent
344 with the variables tested (Table 4, Fig 4). In general, it appears that the planktonic
345 phytoplankton divisions Chlorophyta and Euglenophyta namely *Euglena sociabilis*,
346 *Phacus pleuronectes*, *Chlamydomonas africana* were better able to withstand the
347 effects of vegetable oil than susceptible phytobenthos diatom species. The higher
348 abundance of the benthic diatom species *Fragilaria capucina* ($p \leq 0.05$; $r = 0.970$)
349 and *Navicula viridula* ($p \leq 0.05$; $r = 0.943$) at site 5, correlated positively with the
350 measured positive bottom sediment redox potential at this site in comparison with the
351 negative bottom sediment redox potential at other sampling sites (Tables 2, 3 and 4,
352 Fig 4).

353

354 ***Selenastrum capricornutum* test and chlorophyll concentrations**

355

356 The un-aerated water samples collected from sampling sites 1 to 6 showed no algal
357 growth inhibition. Similar results were obtained for water samples that were
358 constantly aerated during the 72 hour algal bioassay (results not shown) compared to
359 the experimental control containing Milli-Q® water and AAP meduim. All sampled
360 water of the 6 sampling sites stimulated algal growth in the un-aerated bioassays. In

361 some instances the non-sterile samples showed a larger stimulation (110 %) in growth
362 than the sterile samples (Fig. 2). Indeed, after just 24 h of exposure, site 4
363 demonstrated increases in chlorophyll *a* and *b* concentrations in relation to controls
364 (Fig. 3 A), and similar trends were again observed for sites 2, 3, 4 and 6 after 48 h to
365 96 h of exposure (Fig. 3 B, C, D). Of the oil contaminated sites, only site 1 failed to
366 show any real changes in chlorophyll *a* and *b* concentrations with controls at all times
367 of exposure. Site 5, less impacted by the sunflower oil, essentially harbors chlorophyll
368 *a* and *b* contents that were commensurated with controls at all times of exposure.
369 Furthermore, chlorophyll *b* concentrations were higher in comparison with
370 chlorophyll *a* within the first 24 h after exposure of *Selenastrum* cells to sampling
371 water of sampling site 6. However, this phenomenon change after 72 h of exposure
372 when chlorophyll *a* concentrations increased in comparison with chlorophyll *b* in
373 *Selenastrum* cells exposed to the sampled water of sampling site 6.
374
375 **Physical and chemical measurements of oil contamination in water and sediment**
376
377 A distinctive difference was observed between the sunflower oil concentrations in the
378 sediment of sampling sites 1, 2 and 3 incomparison with sites 4, 5 and 6 which were
379 in distance further away from the stormwater inlet (Fig 1). There were a significant
380 difference ($p \leq 0.05$; $r = 0.992$) between the measured variables (BOD, conductivety,
381 Total Nitrogen, Total Posphorus, Redox potencial, ph, DO, sulfide and water column
382 transparency) of sites 1, 2, 4 and 6. The Sunflower oil concentrations measured in the
383 water column also varied between the different sampling sites with highest
384 concentrations at sites 1, 2, 3 and 6 (Table 3). The smaller pore spaces in the fine-
385 textured wetland bottom sediment (average clay particle diameter of 0.1 µm) was not

386 readily penetrated by oil, since high average concentrations (1.76, 1.26, 78.91, 0.165,
387 0.09 and 0.145 mg g^{-1}) of oil were only measured within the first 2 cm of the bottom
388 sediment after which concentrations of oil decline with bottom sediment depth at all 6
389 sampling sites. The absorption of the sunflower oil by clay particles may have
390 induced a decrease in sediment permeability. The decrease in the bottom sediment's
391 permeability could have favoured the formation of anoxic conditions as observed
392 from the average decrease in Eh data in the surface sediment of sites 1, 2, 3, 4 and 6,
393 resulting possibly from the combination of high clay contents in the sediment and the
394 oil adsorption to the particles (Table 3). The sediment of sampling site 3 contains the
395 highest oil concentration (78.91 mg g^{-1}) compared to the other sampling sites. Hence,
396 the lowest concentration of sunflower oil (0.09 mg g^{-1}) and positive Eh data (110 mV)
397 was measured in the bottom sediment of sampling site 5. These observations also
398 concurred with the data of the phytobenthos diversity ($H' = 2.83$) at site 5, indicating
399 that the lowest oil concentration in the sediment at this site coincided with the highest
400 benthic diatom species diversity and abundance of > 5000 cells per cm^2 (Tables 1 and
401 2).

402 A distinctive blackish colour of the sediment (0-5 cm) within the core samples
403 of sampling sites 1, 2, 3, 4 and 6 were observed, as well as a foul smell. The oil
404 concentrations within the water column were higher compared to the sediment at
405 sampling sites 1, 2 and 3 and concurred with the low average DO concentrations at
406 these sites (Table 3). Also, the highest concentration of sunflower oil in the sediment
407 of site 3 (78.91 mg l^{-1}) coincided with the highest negative redox potential (-225 mV)
408 measured at this site. The vertical light extinction measured with the Secchi disc
409 showed low water transparencies at all sites. The highest transparency (57 cm) was
410 measured at sampling site 5 (Table 1). Total nitrogen and phosphorus concentrations

411 measured during the four field trips, consistently decreased (58 % - 91 %) from the
412 inflow (sampling site 1) to the outflow (sampling site 5) of the wetland indicating a
413 nutrient-depletion gradient.
414
415 **BOD, DO and organic matter**
416
417 The BOD values which indicated the amounts of biodegradable organic material
418 (carbonaceous demand) and the oxygen used to oxidize inorganic material such as
419 sulphide and ferrous iron were relatively low in the water column of all the sampling
420 sites except for sites 4 and 5. Higher levels of BOD (144 mg l^{-1}) were measured at
421 site 5 in comparison to the other sampling sites (Table 3). However, we did not
422 observe a steep decline in DO conditions in the water column at site 5 compared to
423 the other sites. Moreover, site 5 had the highest DO concentration (5.3 mg g^{-1})
424 compared to all sites. The sites most affected by relative low average DO were sites 1,
425 2, 3 and 6; these were also the sampling sites with the highest induced corresponding
426 changes in the species composition of affected diatom communities (Table 2). The
427 average % organic matter (dry weight) content of the substrate at sites 1, 2, 3, 4 and 6
428 were higher (4.74 %) than the value of 2.11 % recorded for site 5.
429
430 **pH and redox potential analysis**
431
432 The redox potential analysis of the sediment showed a negative value for all measured
433 sites, except for site 5 with a positive redox potential (110 Mv), which indicated a
434 significant relationship with the higher benthic diatom species abundance (> 5000
435 cells per cm^2) at this sampling site (Table 4). The highest negative redox potential was

436 detected at site 3 in comparison with the other sampling sites (Table 3). The sediment
437 pH did not vary markedly within the first 5 cm depth at all sampling sites and
438 oscillated between 5.9 and 6.2 throughout the study period (Table 3).
439
440 **Discussion**
441
442 When natural seasonal or interhabitat variations in phytoplankton compositions are
443 not well documented, as in the case of the Con Joubert Bird Sanctuary wetland,
444 changes in taxonomic composition can be related to human activities. This can be
445 achieved by comparing shifts in phytoplankton taxonomic composition to
446 environmental changes with autecological characteristics of species, and relating
447 inferred environmental changes to human activities (Oberholster et al. 2007a). The
448 diversity of phytoplankton communities can reflect an entire complex of ecological
449 parameters at a particular site. Such indices are referred to as autecological, because
450 they are based on the autecological characteristics of taxa. Shifts in functional groups
451 of phytoplankton such as different growth forms and divisions of phytoplankton can
452 also indicate an important change in food quality and in habitat structure for
453 invertebrates (Oberholster et al. 2007b). Most wetland phytobenthos is loosely
454 associated with emergent plants (metaphyton), attached to plants or colonized in
455 sediment (Wehr and Sheath, 2003).
456 Phytoplankton represents between 30 and 50 % of primary producer biomass
457 in wetland systems, and their activity is apparent in the large diurnal changes in
458 dissolved O_2 and CO_2 (Wehr and Sheath, 2003). Because of their close connection
459 with water chemistry, phytobenthos help regulate water quality in wetlands, especially
460 phosphorus loading from urban runoff (McCormick and Stevenson 1998).

461 Due to the lack of available data in the literature on the exposure of
462 phytoplankton species to vegetable oil spills in freshwater environments, we
463 compared some of the data generated from this study to earlier data on phytoplankton
464 exposure to crude oil spill in marine environments. Observations of the effects of
465 crude oil spillages all over the world have indicated the remarkable ability of species
466 of Chlorophytes to invade areas from which other species have been eliminated
467 (O'Brien and Dixon 1976). This phenomenon was also observed in our study where
468 *Chlamydomonas africana* was the dominant post spilled species at all sampling sites.
469 The occurrence of this phenomenon was possibly due to reduction of competition
470 after the elimination of sensitive species which enabled tolerant specimens to
471 maximize their reproductive potential as noted from the high abundance of young and
472 mature resting zygotes of *Chlamydomonas africana.* A second possible explanation
473 for the dominance of *Chlamydomonas* species at sampling sites 1, 2, 3, 4 and 6 were
474 the ability of this genus to make use of algal phagotrophy (*i.e.* species that depend
475 more on ingested bacteria than on photosynthesis) under low light conditions as
476 observed in this study (Bird and Kalff 1989) and this phenomenon may also explain
477 the low levels of BOD measured at these sites, notwithstanding the higher organic
478 matter measured in the sediment of these sites. The higher levels of BOD measured at
479 site 5 in comparison to the other sampling sites can also be related to the absence of
480 the macrophyte species *Typha capensis* at this site. In a previous study by Masoko et
481 al. (2008) they observed that the macrophyte *Typha capensis* contained antibacterial
482 properties which could have affected microbial activities at the other sampling sites in
483 our study. A second explanation for the higher BOD at site 5 was possibly due to
484 microbial processes utilizing the oxygen from the water column during degradation
485 processes of the sunflower oil.

486 McCauley (1966) reported that certain phytoplankton species can show
487 tolerance to effects of a large spill of bunker fuel and subsequent deposition of its
488 residues in underlying sediment. In this oil spill incident a persistent but slowly
489 diminishing film formed on the surface of the Muddy River, near Boston, causing
490 various species of *Oscillatoria, Chlamydomonas, Closterium, Fragilaria, Navicula*
491 and *Euglena* to thrive in regions of the highest pollution. These findings are
492 concurrent with observations from this study, except for certain diatom species that
493 were in low abundance at sampling sites with high sunflower oil concentrations in our
494 study. The species *Euglena sociabilis* and *Phacus pleuronectes* that were observed in
495 relative high abundance at sampling sites 1, 2, 3, 4 and 6 in our study are generally
496 associated with polluted water and with water in which organic material is suspended
497 (Canter-Lund and Lund, 1995).

498 The low phytobenthos species abundance at sampling sites 1, 2, 3, 4 and 6
499 with a low diversity index in comparison with site 5 was possibly caused by the oil
500 components (oil in the bottom sediment) adhering to the same substrate particles
501 colonized by these species, as well as surfaces of leaves and stems of the marginal
502 vegetation contaminated by sun flower oil components. In field studies by ZoBell
503 (1964) he observed that the thin films of oil on freshwater do not seem to kill diatoms
504 in underlying layers, but rather affect reproduction. Although, it is known that the
505 growth of diatom species can be limited by a low supply of silica, concentrations as
506 low as 0.2 mg l^{-1} – much less than measured in the studied wetland – should be
507 sufficient for diatom reproduction and therefore seem not to be the reason for the low
508 abundance of diatoms observed at the 6 sampling sites (Willén 1991). The higher
509 abundance of the filamentous cyanobacteria *Anabaena flos-aquae* at site 5 can
510 possibly be related to the low total nitrogen concentration at this site in comparison

511 with the other sampling sites, since this species is a nitrogen fixer (Gaur and Singh,
512 1990). Scott et al. (2005) observed in their study on a fresh water mash, a nutrient-
513 depletion gradient between the inflow and outflow, which is in relationship with data
514 generated in our study. Their results suggested that wetlands may display spatial
515 heterogeneity of specific nutrient limiting phytoplankton due to a nutrient-depletion
516 gradient. However, the higher light transparency measured in the water column of
517 sampling site 5 may also have played a considerable role in the abundance of this
518 species, since light availability is a major contributing factor in controlling nitrogen
519 fixation, which is an energy-demanding process.

520 Evidence from previous marine vegetable oil spills indicated that the spilled
521 oil may undergo polymerisation and persist for up to six years in the environment,
522 however this phenomenon was not observed in our study within the first 60 days after
523 the spill (Mudge 1997). The growth stimulation and changes that occurred after 72 h
524 between chl *a* and *b* concentrations in the *Selenastrum* bioassay were possibly due to
525 adaptive responses of new generations of *Selenastrum* cells to the sunflower oil within
526 the tested water. Hence, the observed high abundance of *Chlamydomonas africana* at
527 sampling sites 1, 2, 3, 4 and 6 can also be related to the possible adaptive responses
528 and the dominance of this species. The reasons are also found in the different
529 responses of phytoplankton on the frequency of disturbances or changes in abiotic
530 resource conditions at different time scales (Reynolds 1984). These different time
531 scales are (1) shorter than one generation time induce physiological responses, (2)
532 frequencies between 20 and 200 h interact with the phytoplankton growth rate, and (3)
533 disturbances at up to 10 days intervals can initiate a successional sequence in
534 phytoplankton development (Reynolds 1984).

535 We suggest from the data generated in this study that adaptation to certain
536 environmental conditions may have played a major role in the absence of less tolerant
537 phytoplankton species (Tezanos Pinto et al. 2006) e.g. the reproduction of benthic
538 diatom species were possibly affected by the high sun flower oil concentrations in the
539 surface layer of the sediment as observed by ZoBell (1964) or by the low light
540 transparency. However, according to Jørgensen (1969) and Harris (1973) diatoms are
541 organisms able to efficiently photosynthesize at low light intensity.

542

543 The non-sterile samples that showed a larger stimulation in growth than the sterile
544 samples in the 72 hour algal bioassay conducted on the wetland water column water
545 can possibly be explained by an increase in growth of other algae species and
546 microorganisms that were present in the wetland water. This stimulation was
547 particularly high in the case of the undiluted water sample taken from sampling site 4.
548 The sample from sampling site 5 which exhibited the lowest growth stimulation
549 within 72 h may be linked to the lowest total nitrogen measured at this site. From the
550 data generated in this study it is evedent that the intracellular level of chlorophyll *a*
551 and *b* measurements as physiological variables were a more sensitive indicators of the
552 adverse effect of sunflower oil than the algal bioassay. Observations from the algal
553 bioassay show that intracellularly the exposed *Selenastrum* cells expressed
554 physiological changes within their photosystems thereby suggesting some degree of
555 stress owing to oil contaminated water.

556 Furthermore, the higher chl *b* concentrations in comparison with chl *a* within
557 the first 24 h after exposure of *Selenastrum* cells to sampling water of sampling site 6
558 were possible due to the fact that chl *a* which is a component of the peripheral antenna
559 complexes may have been altered by the presence of sunflower oil (Anderson 1986).

560 These antenna complexes show controlled changes in adapting to various growth
561 conditions, enabling optimal utilization of available light. However, it is known that
562 the chl *a* to *b* concentrations are higher in high-light growth conditions than in low-
563 light growth conditions, which is accompanied by larger size of complexes in low-
564 light conditions (Björkman et al. 1972). Thus, the regulation of chl *b* synthesis is an
565 important factor for the mechanisms of adaptation of algae to various light intensities
566 which may have been affected by the presence of sunflower oil within the water
567 column causing a change in light intensity. In a study conducted by Reger and Krauss
568 (1970) on the green algal *Chlorella vannielii*, energy demand in the form of adenosine
569 5′-triphosphate (ATP) was strikingly greater when chl *b* concentrations were low
570 (Reger and Krauss 1970). Thus, the accelerated respiration provides the required ATP
571 for the dark reactions of photosynthesis. Therefore, the level of chl *b* appears to reflect
572 a regulatory device in governing cyclic and noncyclic photophorylation (Reger and
573 Krauss 1970). Such reports support our hypothesis that the energy demand for ATP
574 and tempo of respiration which was much lower at the higher chl *b* to *a* concentrations
575 could have had an affect on the cyclic and noncyclic photophorylation of the
576 *Selenastrum* cells within the first 48 h of the algal bioassay. This observation is also
577 supportive to findings by McKee and Wolf (1971) who suggested from their study
578 that the alterations in light intensity and quality below surface layers of oil may inhibit
579 the process of photosynthesis. A study conducted by El-Dib et al. (1997) on the
580 impact of fuel oil on the freshwater alga *Selenastrum capricornutum* revealed that
581 water extracts of fuel oil induced significant changes in chlorophyll *a* content of
582 treated cultures. They observed a general trend with chl *a* content decreasing as the
583 fuel oil concentration in algal cultures increased. However, Tanaka et al. (1998)
584 suggested that chl *a* and *b* may be interconvertible through 7-hydroxymethyl

585 chlorophyll by the chlorophyll cycle in the green algal species *Chlamydomonas*
586 *reinhardtii*. By regulating photosynthetic antenna through interconversion of chl *a* and
587 *b* could be more efficient than their degradation and synthesis, and it would be of
588 great advantage to *Chlamydomonas* species and possibly provide a reason for their
589 dominance in the sunflower oil contaminated freshwater water of this study.

590 The distinctive blackish colour of the sediment (0-5 cm) within the core
591 samples of sampling sites 1, 2, 3, 4 and 6 were possibly due to SO_4^{2-} that was reduced
592 during the microbial oxidation (respiration) of organic matter at these sites, causing
593 gaseous S^{-2} or bisulphide ion (HS-) that was produced to combine with Fe to form
594 almost insoluble precipitates under anoxic conditions at these sampling sites. Under
595 these anoxic conditions Fe (II) may have precipitated as FeS in the presence of
596 enough sulfides at sampling sites 1, 2, 3, 4 and 6, giving the sediment a characteristic
597 black colour. The higher abundance of the benthic diatom species *Fragilaria*
598 *capucina* and *Navicula viridula* at site 5 in relationship with the positive Eh measured
599 at this site served as an indicator (surrogate) of the degree of sediment oxygenation
600 and the suitability of particular bottom sediment for phytobenthos species sensitive to
601 low DO conditions.

602

603 **Conclusion**

604

605 Although the lack of establishment of pre-spill site characterization is a shortfall in
606 this study, it is evident from the data generated that a sunflower oil spill can have an
607 adverse effect on diversity and abundance of certain phytoplankton species in a
608 freshwater wetland environment. The study display spatial heterogeneity of specific
609 nutrient limiting phytoplankton due to a nutrient-depletion gradient between the

610 inflow and outflow. However, the benthic diatom and phytoplankton species diversity
611 were the highest at the site nearest to the outflow. This was also the site with the
612 lowest oil concentrations and highest light transparency in comparison with the other
613 sampling sites; suggesting that nutrient availability at other sampling sites was
614 overshadowed by the higher sunflower oil concentrations and lower light transparency
615 causing a decline in certain phytobenthos species and therefore adversely affected
616 phytoplankton species diversity. From a practical application view point, the data
617 generated in this study can play an important role in post spill vegetable oil restoration
618 actions, especially in the case of using biostimulation – since the application of to
619 high concentrations of fertilizers to stimulate natural microbial activity for
620 biodegrading of vegetable oils in freshwater bodies can cause a further increase in
621 biomass and bloom formation of certain species e.g. toxic filamentous cyanobacteria
622 *Oscillatoria* that can pose a serious threat to the food web structure and functions of
623 fresh water ecological systems.

624

625 **Acknowledgement**

626

627 The authors express their sincere gratitude to the National Research Foundation for
628 provision of funding as well as to Ms JL Slabbert for her input. The authors also want
629 to thank the unknown referees for critically reviewing the manuscript and suggesting
630 useful changes.

631

632 **References**

633

634 Anderson JM (1986) Photoregulation of the composition, function, and structure of
635 thylakoid membranes. Annual. Review of Plant Physiology 37: 93-136.
636 Baker AL, Baker KK, Tyler PA (1985) A family of pneumatically-operated thin layer
637 samplers for replicate sampling of heterogeneous water columns. –
638 Hydrobiologia 22: 107-211.
639 Björkman O, Boardman NK, Anderson JM, Thorne SW, Goodchild DJ, Pyliotis NA
640 (1972) Yearbook, Carnegie Inst. Washington USA 71: 115-135
641 Begg GW (1990) Policy proposals for the Wetlands of Natal and Kwazulu. Natal
642 town and regional planning commission. Report 75. Pietermaritzburg. Z.A.
643 Bird DF, Kalff J (1989) Bacterial grazing by planktonic algae. Science 231: 493-495
644 Breen CM, Begg GW (1989) Conservation status of Southern African wetlands. In:
645 Biotic Diversity in Southern Africa: Concepts and Conservation. Huntley B.J.
646 (ed) Conservation Biology 14: 1490-1499
647 Brower JE, Zar JH, von Ende CN (1990) Field and laboratory methods for General
648 Ecology. WMC Brown publishers, San Diego, USA, pp. 1- 237
649 Bucas G, Saloit A (2002) Sea transport of animal and vegetable oils and its
650 environmental consequences. Marine Pollution Bulletin 44: 1388-1396
651 Carter-Lund H, Lund JWG (1995) Freshwater Algae – Their Microscopic World
652 Explored. Biopress Ltd. pp. 1-360
653 Clesceri LS, Greenberg AE, Eaton AD (1998) Standard Methods for the Examination
654 of Water and Wastewater, American Public Health Association (APHA),
655 Washington, DC.
656 Cornish A, Battersby NS, Watkinson RJ (1993) Environmental fate of mineral,
657 vegetable and transesterified vegetable oils. Pesticits and Science 37: 173-178

658 Code of Federal Regulations (1994) Part 136-Guidelines Establishing Test Procedures
659 for Analysis of Pollutants, 40 CFR, Part 136, Method 413.1, US Government
660 Printing Office, Washington, DC.

661 de Tezanos Pinto P, Allende L, O'Farrell I (2007) Influence of free-floating plants on
662 the structure of a natural phytoplankton assemblage: an experimental approach.
663 Journal of Plankton Research 29: 47-56

664 EL-Dib MA, Abou-waly HF, EL-naby AH (2001) Fuel oil effects on the population
665 growth, species diversity and chlorophyll (*a*) content of freshwater microalgae.
666 International Journal of Environmental Health Research 11: 189-197

667 Gaur JP, Singh AK (1990) Growth, photosynthesis and nitrogen fixation of Anabaena
668 doliolum exposed to Assam crude extract. Bull Environ Contam Toxicol 44:
669 494-500

670 Groenewold JC, Pico RF, Watson KS (1982) Comparison of BOD relationships for
671 typical edible and petroleum oils. Journal of the Water Pollution Control
672 Federation 54: 398-405

673 Hauer FR, Lamberti GA (2006) Methods in Stream Ecology. Academic Press, San
674 Diego, California USA, pp 1- 877

675 Hartung R (1967) Energy metabolism in oil-covered ducks. Journal of Wildlife
676 Management 31: 798-804

677 Harris GP (1973) Diel and annual cycles of net plankton photosynthesis in Lake
678 Ontario, J Fish. Res. Board Can. 30: 1779-1787

679 Hindak F (1990) Biologicke Prace 5: 209

680 Kalff J (2002) Limnology: Inland water ecosystems. Prentice Hall, , New Jersey, pp
681 1- 535

682 McCauley RN (1966) The biological effects of oil pollution in a river. Limnology and
683 Oceanography 1: 475-486
684 McKee JE, Wolf HW (1971) Water Quality Criteria, 2nd ed., California State Water
685 Quality Contr. Bd, Publ, 3-A
686 McKelvey R, Robertson W, Whitehead PE (1980) Effects of non-petroleum spills on
687 wintering birds near Vancouver. Marine Pollution Bulletin 11: 169-171
688 Mitsch WJ, Gosselink JG (1993) Wetlands, 2nd ed., Van Nostrand Reinhold Company
689 Inc., New York, pp 393-414
690 Morant PD (1983) Wetland classification: towards an approach for southern Africa.
691 Limnological Society of South Africa 9: 76-84.
692 Mudge SM, Salgado M, East J (1993) Preliminary investigations into sunflower oil
693 contamination following the wreck of the M.V. Kimya. Marine Pollution
694 Bulletin 26: 40-44
695 Mudge SM (1995) Deleterious effects from accidental spillage of vegetable oils. Spill
696 Science and technology Bulletin 2: 187-191
697 Mudge SM (1997) Can vegetable oils outlast mineral oils in the marine environment?
698 Marine Pollution Bulletin 34: 213
699 Nygaard G, Komarek J, Kristiansen J, Skilberg O (1986) Taxonomic designations of
700 the bioassay alga, NIVA-CHL I (*Selenastrum capricornutum*) and some related
701 strains. Opera Botanica 90: 1-46
702 Oberholster PJ, Botha A-M, Cloete TE (2005) Using a battery of bioassays, benthic
703 phytoplankton and the AUSRIVAS method to monitor long-term coal tar
704 contaminated sediment in the Cache la Poudre River, Colorado. Water Research
705 39: 4913-4924

706 Oberholster PJ, Botha A-M., Cloete TE (2007a) Ecological implications of artificial
707 mixing and bottom-sediment removal for a shallow urban lake, Lake Sheldon,
708 Colorado. Lakes & Reservoirs: Research and Management 12: 73-86

709 Oberholster PJ, Botha A-M, Cloete ET (2007b) Biological and chemical evaluation of
710 sewage water pollution in the Rietvlei Nature Reserve wetland area, South
711 Africa. Environmental Pollution 156: 184-192

712 Oberholster PJ, Slabbert JL, McMillan PH, (2008) Diagnostic risk assessment of a
713 wetland area near the Nola facility, Randfontein after the occurrence of a
714 sunflower oil spill. CSIR Report No. CSIR/NRE/WR/ER/2008/0016/C. CSIR,
715 Pretoria South Africa, pp 1- 44

716 Patrick R, Reimer CW (1975) The diatoms of the United State exclusive of Alaska
717 and Hawaii, vol. 2, Part 1. Monograph 13, Academy of National Sciences,
718 Philadelphia, PA, pp 175

719 Pereira MG, Mugde SM, Latched J (2002) Consequences of linseed oil spills in salt
720 marsh sediments. Marine Pollution Bulletin 44: 520-533

721 Pezeshski SR, DeLaune RD, Patrick WH (1989) Effect of fluctuating rhizosphere
722 redox potential on carbon assimilation of *Spartina alterniflora*. Oecologia 80:
723 132-135

724 Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate
725 extinction coefficient and simultaneous equations for assaying chlorophyll *a* and
726 *b* extracted with four different solvents: verification of the concentration of
727 chlorophyll standards by atomic absorption spectrometry. Biochimica
728 Biophysica Acta 975: 384-394

729 Reger BJ, Krauss RW (1970) The Photosynthetic Response to a Shift in the
730 Chlorophyll *a* to Chlorophyll *b* ratio of *Chlorella*. Plant Physiology 46: 568-575

731 Rigger D (1997) Edible oils: Are they really that different? In: Proceedings,
732 Internasional Oil Spill Conference, American Pretoleum Institute, Washington,
733 DC, pp 59-61
734 Reynolds CS (1984) Phytoplankton periodicity: the interactions of form, function and
735 environmental variability. Freshwat Biol 14: 111-142
736 Ross P, Jarry V, Sloterdijk H (1988) A rapid bioassay using the green algal
737 *Selenastrum capricornutum* to screen for toxicity in St. Lawrence River
738 sediment elutriates. In: Cairns J, Pratt JR (eds) Functional Testing of Aquatic
739 Biota for Estimating Hazards of Chemicals. ASTM STP 988. American Society
740 for Testing and Materials, Philadelphia, USA, pp 68-73
741 Scott JT, Doyle RD, Filstrup CT (2005) Periphyton nutrient limitation and nitrogen
742 and nitrogen fixation potential along a wetland nutrient-depletion gradient.
743 Wetlands 25: 339-448
744 Shannon CE, Weaver W (1949) The Mathematical Theory of Communications.
745 University of Illinois Press, Urbana, Illinois, USA
746 Slabbert JL (2004) Methods for direct estimation of ecological effect potential
747 (DEEEP). Water Research Commision Report No. K5/1313/1/04, Pretoria,
748 South Africa, pp 1-100
749 Jørgensen EG (1969) The adaptation of plankton algae. IV. Light adaptation in
750 different algal species. Plant Physiol 19: 1307-1315
751 Sun M-Y, Wakeham SG, Lee C (1997) Rates and mechanisms of fatty acid
752 degradation in oxic and anoxic coastal marine sediments of Long Island Sound,
753 New York, USA. Geochimica et Cosmochimica Acta 61: 341-355

754 Tanaka A, Ito H, Tanaka R, Tanaka NK, Yoshida K, Okada K (1998) Chlorophyll *a*
755 oxygenase (*CAO*) is invoved in chlorophyll *b* formation from chlorophyll *a*.
756 Proceedings National Academic of Science, USA, Vol. 95, pp 12719-12723
757 Taylor JC, Harding WR, Archibald CGM (2007) An illustrated guide to some
758 common diatom species from South Africa. Water Research Commission,
759 Report TT 282/07, Pretoria, pp 1-178
760 Ter Braak CJF (1986) Canonical correspondence analysis: a new eigenvector
761 technique for multivariate direct analysis, Ecology 67: 1167-1179
762 U.S. Department of Commerce (USDOC), National Oceanic and Atmospheric
763 Administration (NOAA) (1996) Damage Assessment and Restoration Program.
764 Injury Assessment: Guidance Document for Natural Resources Damage
765 Assessment under the Oil Pollution Act of 1990. Silver Spring, Maryland,
766 Appendix C, Oil behavior, Pathways, and Exposure, pps. C-1-24 and Appendix
767 D, Adverse effects from oil, pp D-1-69, Augustus, 1996.
768 Van Vuuren S, Taylor JC, Gerber A, Van Ginkel C (2006) Easy identification of the
769 most common freshwater algae. North-West University and Department of
770 Water Affairs and Forestry, Pretoria, South Africa, pp 1-200
771 Wehr JD, Sheath RG (2003) Freshwater Algae of North America: Ecology and
772 Classification. Academic Press, Massachusetts, USA, pp 1-834
773 Willen E (1976) A simplified method of phytoplankton counting. Br J Phycol 11:
774 265-278.
775 Willén E (1991) Planktonic diatoms – an ecological review. Algological Studies 62:
776 69-106.

777 Zobell CE (1964) The occurrence, effects, and fate of oil polluting the sea. In
778 International Conference on Water Pollution Research, London, September
779 1962, pp 85-109
780 Zoun PEF, Baars AJ, Boshuizen RS (1991) A case of seabird mortality in the
781 Netherlands caused by spillage of nonylphenol and vegetable oils, winter
782 1988/1989. Sula 5:101-103

783 **Table 1.** Aquatic marginal vegetation and biotopes of each sampling location in the Con Joubert Bird Sanctuary wetland.

784

Sampling location	**Site1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**
Water depth (metres)	1.0	0.9	1.3	0.95	0.9	0.85
Biotopes	Clay	Clay	Clay	Clay	Clay	Clay
Marginal emergent vegetation	*Typha capensis*, *Phragmites australis*, *Schoenoplectus brachyceras*, *Cyperus marginatus*, *Persicaria lapathifolia*	*Typha capensis*, *Phragmites australis*	*Typha capensis*, *Phragmites australis*, *Persicaria lapathifolia*	*Typha capensis*, *Persicaria lapathifolia*, *Phragmites australis*,	*Cyperus marginatus*, *Phragmites australis*, *Schoenoplectus paludicola*	*Typha capensis*, *Phragmites australis*
Floating leave Plants			*Spirodela* spp.	*Azolla pinnata*		

785

786

787 **Table 2.** Comparison of the phytoplankton at the six sampling sites in Con Joubert freshwater wetland (n = 4). The numbers (1-4) represent the
788 maximum frequencies of the phytoplankton taxa: where 1 ≤ 250, 2 = 251-1000, 3 = 1001-5 000 and 4 = 5 001-25 000 cells l^{-1} and cells. cm^{-2} in
789 the case of phytobenthos taxa = (B).

Algal division	Genus and species	site 1	Site 2	site 3	site 4	site 5	site 6
Bacillariophyta	*Aulacoseira granulata* (B)	1	0	0	0	0	0
	Aulacoseira thwaites(B)	0	0	0	2	0	0
	Amphora pediculus	0	0	1	0	2	1
	Cocconeis placentula (B)	0	0	0	0	1	0
	Gomphonema parvulum	0	0	0	0	1	0
	Fragilaria crotonensis	0	0	1	0	2	0
	Fragilaria ulna (B)	1	2	1	1	2	1
	Fragilaria capucina (B)	0	0	0	1	3	1
	Navicula viridula (B)	0	0	1	0	2	0
	Nitzschia umbonata	0	2	0	0	1	0
	Pinnularia viridiformus	1	1	1	1	0	1
Euglenophyta	*Euglena sociabilis*	4	3	4	3	2	4
	Phacus pleuronectes	3	3	3	4	2	3
	Phacotus lenticularis	2	0	0	0	1	0
	Trachelomonas intermedia	3	3	3	4	2	4
	Trachelomonas armata fa. Inevoluta	2	0	0	0	2	0
Chlorophyta	*Chlamydomonas africana.*	4	4	4	3	2	4
	Chlamydomonas africana, young and mature resting zygotes	4	4	4	4	3	3
	Closterium lineatum	0	0	1	0	3	0
	Volvox rousseletii	0	0	0	0	1	1
	Scenedesmus dimorphus	0	0	1	0	2	0
Cyanophyta	*Oscillatoria princeps*	0	1	3	2	0	2
	Anabaena flos-aquae	0	1	0	1	3	1
Shannon Index (*H*)		1.91	1.62	1.57	1.69	2.83	1.74

790

791 **Table 3.** Comparison of the average physical and chemical parameters recorded at six sampling sites in the Con Joubert freshwater wetland (n =

792 4) before biostimulation of natural microbial activity takes place.

793

Sampling sites	**1**	**2**	**3**	**4**	**5**	**6**
BOD in water column	24 (± 8)	24 (± 11)	30 (± 9)	20 (± 11)	144 (± 16)	48 (± 9)
DO in water column as mgl^{-1}	1.3 (± 1)	1.6 (± 1.1)	2 (± 0.9)	2.1 (± 1)	5.3 (± 2)	2.5 (± 0.6)
pH value at 20 ºC in water column	6.9 (± 0.2)	7.2 (± 0.4)	7.4 (± 0.2)	7.3 (± 0.5)	7.1 (± 0.3)	7.4 (± 0.2)
pH value at 20 ºC in (0-5 cm) depth sediment	5.7 (± 0.7)	5.5 (± 0.3)	5.3 (± 0.1)	5.9 (± 0.4)	6.1 (± 0.3)	6 (± 0.6)
Eh as mV in (0-5 cm depth) sediment	-190 (± 10)	-187 (± 8)	-225 (± 13)	-211 (± 15)	110 (± 27)	-209 (± 12)
Total Nitrogen (TN) in water column ($mg\ l^{-1}$)	0.37 (± 0.6)	0.34 (± 0.4)	0.36 (± 0.3)	0.14 (± 01)	0.037 (± 0.05)	0.29 (± 0.5)
Total Nitrogen (TN) in sediment ($mg\ kg^{-1}$)	52 (± 9)	25 (± 8)	18.7 (± 4)	21 (± 5)	21 (± 7)	39 (± 3)
Total Phosphorus (TP) in water column ($mg\ l^{-1}$)	1.81 (± 0.9)	1.76 (± 0.5)	1.76 (± 0.8)	1.43 (± 0.2)	0.643 (± 0.09)	0.448 (± 0.06)
Total Phosphorus (TP) in sediment ($mg\ kg^{-1}$)	25 (± 3)	14 (± 7)	10.4 (± 2)	20 (± 8)	8.2 (± 1)	13.7 (± 5)
Conductivity in water column ($ms\ m^{-1}$)	44 (± 10)	37.1 (± 8)	53.5 (± 12)	37.7 (± 14)	30.75 (± 3)	26.6 (± 4)
Temperature in water column (ºC)	21.6 (± 0.4)	23 (± 1)	20.9 (± 0.7)	23.5 (± 0.5)	21.1 (± 0.8)	23.1 (± 0.8)
Total concentration oil ($mg\ g^{-1}$) in surface layer (0-5cm) of sediment	1.76 (± 0.6)	1.26 (± 0.2)	78.91 (± 3.4)	0.165 (±0.04)	0.09 (± 0.02)	0.145 (± 0.09)
Sulfide SO_4^{2-} ($mg\ kg^{-1}$)	7.9 (± 0.2)	5.7 (± 0.8)	102 (± 6.9)	43 (± 2.3)	5 (±0.5)	78 (± 3.5)
Oil within the water column samples ($mg\ l^{-1}$)	2 (± 0.1)	1.8 (± 0.3)	81.5 (± 0.9)	0.8 (± 0.4)	1.3 (± 0.2)	14.7 (± 0.7)
Secchi Disc reading (cm)	17	15	23	19	57	21
Organic matter (%) dry weight in substrate	5.14	4.92	5.87	3.47	2.11	4.33

794

795

796

797 **Table 4.** Values of Pearson's correlation coefficients ($p < 0.05$) between phytoplankton biomass and environmental variables (n.s. = not
798 significant)
799

Species	BOD	DO	pH water	pH sediment	Eh	TN water	TN sediment	TP water	TP sediment	Conductivity	Tempart ure	Total oil Surface Layer	Sulfide	Oil Water Column	Secchi Disc	Organic Matter
C. africana	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.967**	**0.856**	**0.979**	**0.967**	n.s.
O. princeps	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.963**	n.s.	**0.968**	**0.935**	n.s.
A. flos-aquae	**0.978**	**0.958**	n.s.	n.s.	**0.994**	**-0.819**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
F.ulna	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
F.capucina	**0.981**	**0.970**	n.s.	n.s.	**0.990**	**-0.847**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**-0.830**
N.viridula	**0.949**	**0.936**	n.s.	n.s.	**0.943**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

800
801
802
803
804
805

Figure legends

Figure 1. Map of the Con Joubert Bird Sanctuary freshwater wetland showing the location of six sampling sites and the storm water inflow inlets. Inset shows the location of the map area in South Africa.

Figure 2. Bioassay indicating growth stimulation (%) of sterile and non-sterile *Selenastrum capricornutum* cells after 72 hours incubation with undiluted water (100 % concentration of oil contaminated water) of the six different sampling sites. Control = 0 %.

Figure 3. A, B, C and **D.** Changes in chlorophyll *a* and *b* concentrations of *Selenastrum capricornutum* cells after 24; 48, 72 and 96 hours incubation with undiluted wetland water (100 % concentration of oil contaminated water) of the six different sampling sites. * Indicates a significance from the control by Turkey test ($p < 0.05$)($n = 5$).

Figure 4. Diagram (Canonical Correlation Analysis) of dominant species for the six sampling sites in relation to environmental variables. Vectors grouped together indicate the correlation between phytoplankton species and physiochemical variables are statistically significant ($p < 0.05$). Y1 = phytoplankton species (red); Y2 = physiochemical variables (green).

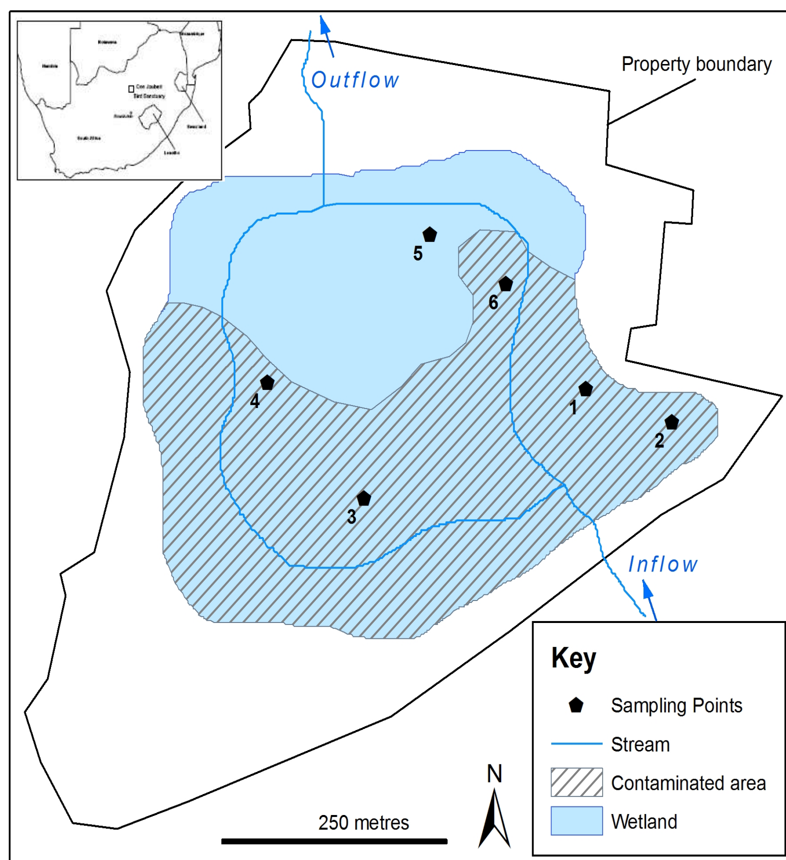

Figure 1

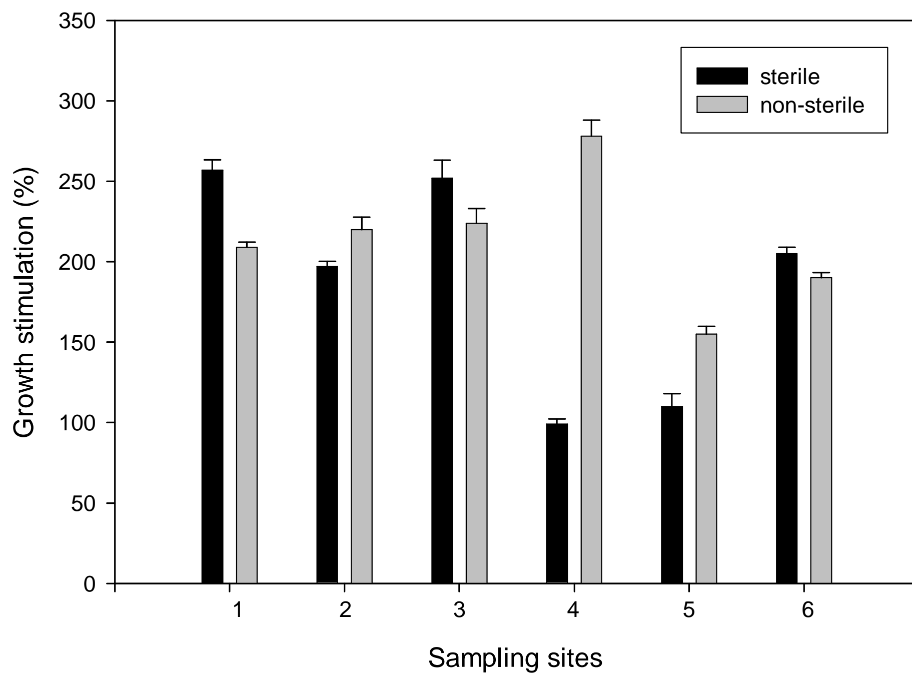

Figure 2

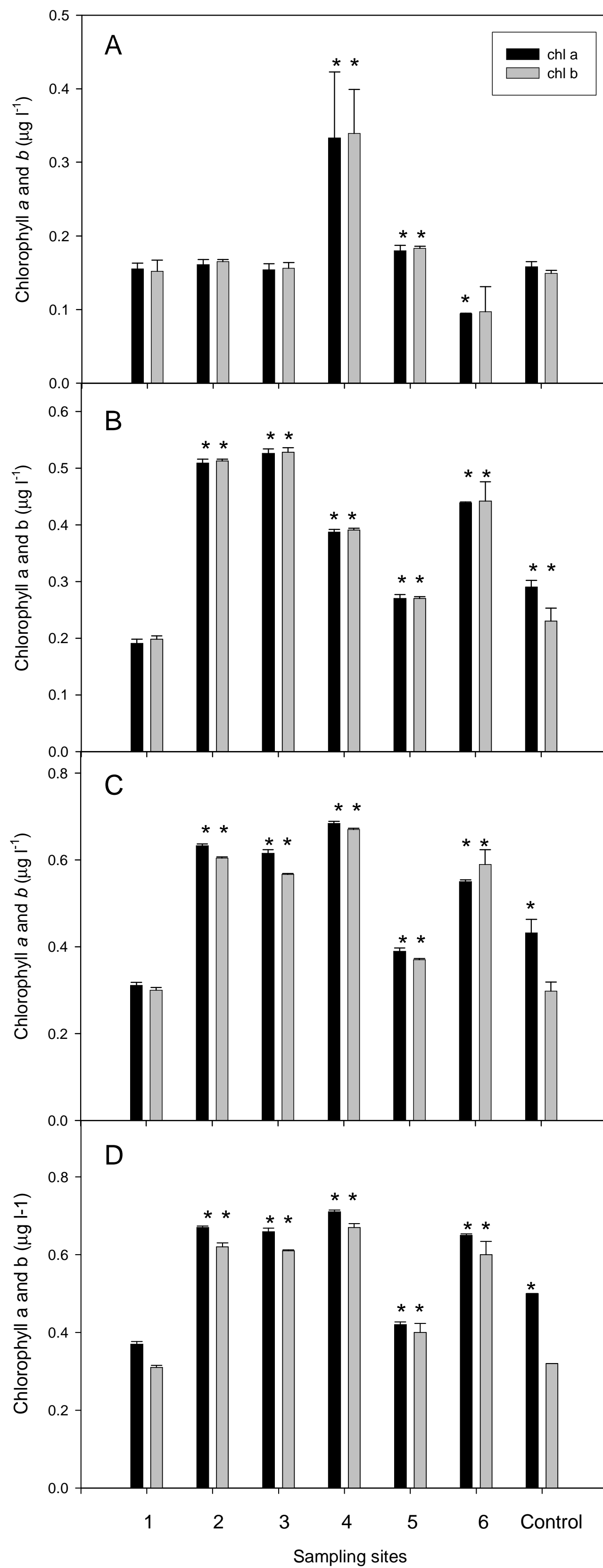

Figure 3