Korean J. Limnol. 40 (4): 520~526 (2007)

Inhibition of Aquatic Vascular Plants on Phytoplankton Growth
II. Algal Growth Experiments with Water and Plant Extracts from Submerged Macrophytes

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To select submerged macrophytes to suppress growth of *Microcystis aeruginosa* through releasing allelochemicals, we conducted growth experiments with water from patches of submerged macrophytes and with aqueous extracts of those submerged macrophytes. In the first experiment, growth rates of *M. aeruginosa* decreased as biomass of *Myriophyllum spicatum* and *Hydrilla verticillata* increased. In the second experiment, *M. aeruginosa* showed approximately 50% growth reduction with extracts from *M. spicatum* and 24% reduction with extracts from *Ottelia alismoides*. Both *M. aeruginosa* growth experiments with water and plant extracts suggest that *M. spicatum* would be the best candidate to reduce *M. aeruginosa* growth.

Key words : submerged macrophytes, *Microcystis aeruginosa*, allelopathic substances, inhibition of algal growth

INTRODUCTION

Recently, many Korean freshwater ecosystems have become eutrophicated due to rapid industrialization and economic development to cause serious ecological and economical problems. Considerable research interests are focusing on regulating cyanobacterial water blooms using physical, chemical and biological approaches. A recent addition on regulating cyanobacterial water blooms is using aquatic vascular plants, especially submerged plants (Van Donk and de Bunk, 2002). However, lake management studies using aquatic macrophytes are mainly concentrating on nutrient absorption of plants to compete with phytoplankton (Romero *et al.*, 1999; Coveney *et al.*, 2002; Dierberg *et al.*, 2002). A recent study reviewed on the interaction among macrophytes, phytoplankton, and periphyton summarized inhibition mechanisms of macrophyte on phytoplankton as light, temperature, nutrient competition, and allelopathy (Van Donk and de Bunk, 2002). Two most studied submerged macrophytes in regarding to allelopathic inhibition on phytoplankton are *Myriophyllum* (Planas *et al.*, 1981; Gross and Sütfeld, 1994; Gross, 1999; Nakai *et al.*, 2000; Nakai *et al.*, 2005) and *Chara* (Crawford, 1979; Jasser, 1995). In particular, *Myriophyllum spicatum* has been reported to produce several polyphenol compounds and fatty acids to reduce *Microcystis aeruginosa* growth (Nakai *et al.*, 2000; Nakai *et al.*, 2005).

In our previous study, we surveyed 21 water bodies with submerged macrophytes in summer and a reservoir from spring to fall to select candidate plants capable of producing allelopathic substances. We found that chlorophyll *a* to total phosphorus concentration ratios decreased in waters from patches of certain submerged macrophytes: *Myriophyllum spicatum* and *Hydrilla verticillata* as their biomass increased (Joo *et al.*, 2007), In the present study, we attempted more direct approaches: *M. aeruginosa* growth experi-

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ments with water collected from patches of submerged plants and with extracts of submerged macrophytes. Purpose of this study was to provide useful information to select possible candidates to release allelopathic substances to regulate bloom causing *M. aeruginosa* in aquatic ecosystems in Korea.

MATERIALS AND METHODS

1. Study sites and sample collection

We collected submerged macrophytes and waters from their patches from twenty one water bodies in Gyeonggi-do, Chungcheongnam-do and Chungcheongbuk-do in August, 2006 (Joo *et al.*, 2007). Detailed description of those sites may be found in the study by Joo *et al.* (2007). Water samples were collected from the patches of submerged macrophytes using Van Dorn water sampler (Wildco, USA). The water samples were kept at 4°C with ice during transport to the laboratory. Water samples were filtered using GF/C (Whatman, USA) to remove seston and stored at −20 °C until experiments. Submerged macrophytes, such as *H. verticillata, Caratophyllum demersum, Potamogeton macckianus, Limnophila sessilliflora, M. spicatum* and *Chara* spcies, were sampled using a quadrat (0.4×0.4 m) with replications (n=3). Collected plants were dried at room temperature in shade for one week. Dried plant samples in replications were mixed into one composite sample, followed by weighing and powdering using a grinder.

2. Algal culture and extraction of macrophytes

Microcystis aeruginosa was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA (UTEX) and cultured in modified L16 medium (Lindström, 1983) in a temperature-controlled chamber at 24°C and 16 : 8 h light : dark cycle. We modified L16 medium by enriching nitrogen (×12 $NaNO_3$) to optimize *M. aeruginosa* growth. For the present experiment, *M. aeruginosa* cultures were kept in exponential growth phase by weekly subculturing. To ensure high growth rates of *M. aeruginosa* during the experiment, we used *M. aeruginosa* in exponential growth phase 5-7 days after subculture innoculation for the growth experiments.

We prepared aqueous extracts of twenty-four ground plant samples. Samples were extracted in deionized water (1 : 15 w/v) in 250 mL Erlenmeyer flasks (Rice *et al.*, 2005). The flasks were sealed with a parafilm layer and incubated for 24 h in the dark at 4°C with constant shaking at 100 rpm. The slurry was filtered through 2 layers of mesh cloth, and then centrifuged for 10 min at 3,000 rpm. Supernatant was filtered by using GF/C (Whatman, USA) and 0.45 μm membrane filter (Millipore, USA). Extracts were stored at 4 °C until growth experiments.

3. Algal growth experiment with water

A *M. aeruginosa* growth experiment was conducted using 29 water samples from 29 patches of 9 submerged macrophytes for 24 h in order to find the candidate plants producing allelochemical substances. Five plants had multiple water samples (n=3−9) while 4 plants had only one water sample. Ten mL of *M. aeruginosa* were innoculated in modified L16 medium with each water sample (1 : 1 v/v, total volume: 100 mL). The growth experiment was conducted in growth chamber at 24°C and 16 : 8 h light : dark cycle. In control treatment, *M. aeruginosa* were innoculated in modified L16 medium. Growth rates of *M. aeruginosa* were measured using chlorophyll *a* concentration as follows (Eaton *et al.*, 2005) :

$$\text{chlorophyll } a = 11.85 \times OD_{664} - 1.54 \times OD_{647} - 0.08 \times OD_{630} \quad (1)$$

OD_{664}, OD_{647} and OD_{630} indicate optical density at 664, 647 and 630 nm.

M. aeruginosa growth rates were calculated as follow:

$$g = \{Ln(C_t) - Ln(C_0)\}/t \quad (2)$$

where C_t and C_0 are the chlorophyll *a* after t days and at the beginning.

4. Algae growth experiment with plant extracts

A *M. aeruginosa* growth experiment was conducted using 75 experimental units from triplicates of 24 plants and one control for three days. To make a same dissolved organic carbon (DOC) concentration, we measured absorption coefficient at 320 nm (a_{320}) of 24 extracts and diluted each extract to have a_{320} of 9.2. Absorption coefficient (a_{320}) was estimated from absorbance at 320 nm (D) by dividing by the optical path length (r) (Williamson *et al.*, 1999)

$$a_{320}=2.303D/r \quad (3)$$

Microcystis aeruginosa (10% of total solution volume (10 mL)) were cultured in modified L16 medium with plant extracts diluted to have the same DOC level at 24°C and a light intensity of 76 μmol m^{-2} s^{-1} under 16 : 8 h light : dark cycle. In control treatment, *M. aeruginosa* were innoculated in modified L16 medium. After 3 days, chlorophyll *a* was measured using fluorometer (Trilogy, Turner designs, USA) according to EPA Method 445.0, except for acidification step. *Microcystis aeruginosa* growth rates were calculated using equation (2). Standard t-tests between control and treatments were performed with S-Plus

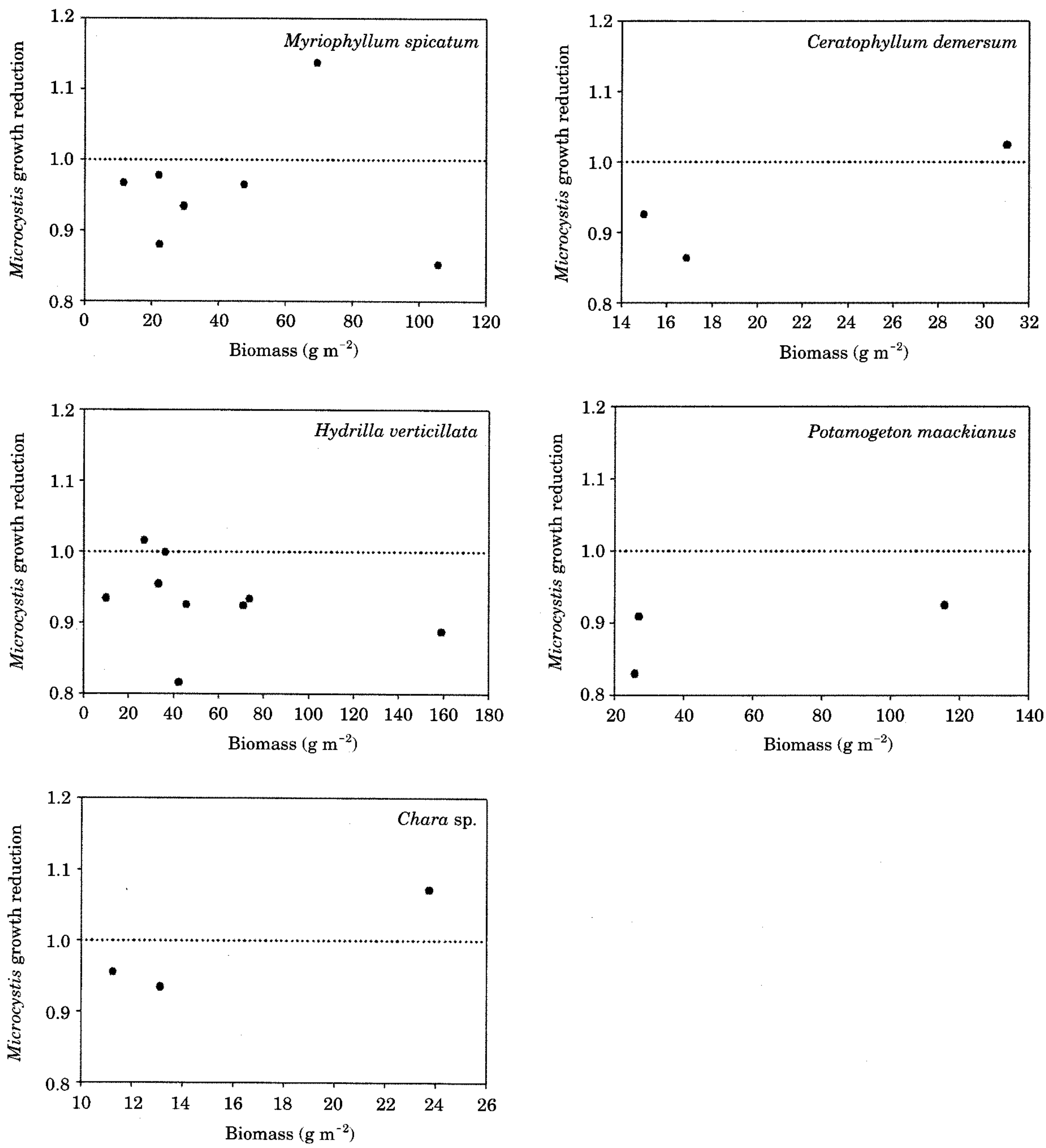

Fig. 1. Relationship between *Microcystis* growth reduction and biomass of 5 submerged plants in different water bodies in August.

Table 1. Summary of *M. aeruginosa* growth experiment with water collected in the patches of submerged macrophytes occurred from only one site. *Microcystis* growth reduction was calculated as a ratio of growth rates in the treatment to growth rates in the control.

Site	Species	Biomass (g m^{-2})	*Microcystis* growth reduction
Gosam Reservoir	*Ottelia alismoides*	25.6	0.85
Sewol Reservoir	*Limnophlia sessiliflora*	3.8	0.96
Dogok Reservoir	*Potamogeton berchtoldii*	25.8	0.99
Eoui Pond	*Potamogeton malaianus* var. *latifolius*	44.0	1.00

6 for Windows (Insightful Corp., USA).

RESULTS

In both experiments with water and extracts, *M. aeruginosa* in control treatment showed very high growth rates between 0.7-1.0 day^{-1}. Most water samples reduced the growth of *M. aeruginosa* compared with control (Fig. 1). *Myriophyllum spicatum* and *H. verticillata* showed a tendency to decrease *M. aeruginosa* growth as plant biomass increased. However, *C. demersum* and *Chara* sp. showed a tendency to facilitate *M. aeruginosa* growth as plant biomass increase while *P. maackianus* did not show any relationship between *M. aeruginosa* growth and plant biomass.

There are four water samples collected from only one site (Table 1). Three submerged macrophytes except *Ottelia alismoides*, did not show significant suppression effect. Although *O. alismoides* reduced approximately 15% growth rate compared with control, only one observation restricted us to detect any pattern in relationship between *M. aeruginosa* growth and their biomass.

Similar inhibition pattern was found in *M. aeruginosa* growth experiment with plant extracts. *Microcystis aeruginosa* showed approximately 50% growth reduction with extracts from *M. spicatum*, 24% reduction with extracts from O. alismoides and 12% reduction with extracts from *P. maackanus*. *Ceratophyllum demersum* and *H. verticillata* suppressed growth rate of *M. aeruginosa* below 10% (Table 2). To see the regional difference, we examined *M. aeruginosa* growth rates with extracts from same submerged macrophyte species and conducted standard t-tests between control and treatments (Fig. 2). *M. spicatum* collected from all sites reduced growth rates of *M. aeruginosa* while other plants did not show clear inhibition pattern.

Table 2. *Microcystis aeruginosa* growth reduction cultured with extracts of submerged plants. *Microcystis* growth reduction was calculated as a ratio of growth rates in the treatment to growth rates in the control. Numerals in parentheses indicate standard errors of means.

Species	Number of sites	*Microcystis* growth reduction
Myriophyllum spicatum	6	0.50 (0.038)
Ottelia alismoides	1	0.76 (0.091)
Potamogeton maackianus	3	0.88 (0.060)
Caratophyllum demersum	3	0.90 (0.026)
Hydrilla verticillata	10	0.92 (0.036)
Limnophila sessiliflora	1	1.05 (0.048)

DISCUSSION

Our results indicate that *M. spicatum* is the best candidate to reduce *M. aeruginosa* growth by producing allelochemical substances (Table 2). Both *M. aeruginosa* experiments with water and extracts, *M. spicatum* appear to reduce *M. aeruginosa* growth. The present study supports our previous work on field evidence for phytoplankton suppression which found that phytoplankton in the patches of *M. spicatum* showed less standing crops than predicted based on total phosphorus (Joo *et al.*, 2007). Our study also support the notion that *M. spicatum* is able to suppress *M. aeruginosa* through allelochemical interactions (Planas *et al.*, 1981; Gross and Sütfeld, 1994; Gross, 1999; Nakai *et al.*, 2000; Nakai *et al.*, 2005). Our results indicate that *M. aeruginosa* may also play important roles in regulating cyanobacteria *M. aeruginosa* in aquatic ecosystems in Korea. However, our results from the growth experiment with water did not support that *Chara* species may be another candidates to

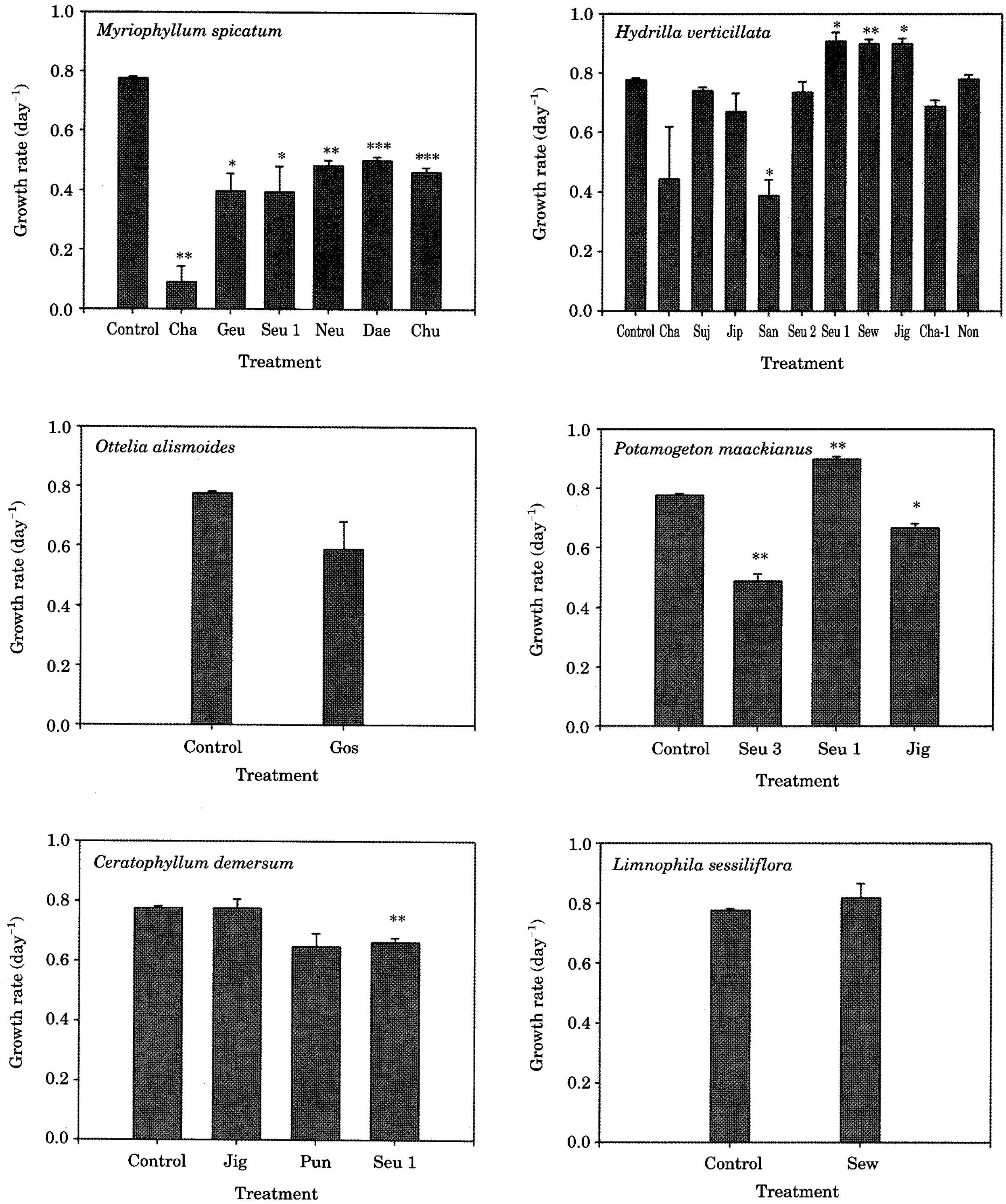

Fig. 2. Relationship between growth rate of *M. aeruginosa* (day^{-1}) and plant extracts from various sites. A *M. aeruginosa* growth experiment was conducted using 3 replications from each plant extract. Cha (Changgi Reservoir), Cha-1 (Changgi Reservoir's other patch) Geu (Marshy land, Geumdae-ri), Seu 1 (Seung-un 1 Reservoir), Seu 2 (Seung-un 2 Reservoir), Seu 3 (Seung-un 3 Reservoir), Neu (A pond for rice fields, Neungnae-ri), Dae (Daesung Reservoir), Chu (Chunsandong Reservoir), Suj (Back marsh, Sujong-myeon), Jip (Jipo Reservoir), San (Sangchun Reservoir), Sew (Sewol Reservoir), Jig (Jigok Reservoir), Non (Nonsan Reservoir), Gos (Gosam Reservoir) and Pun (Pungjun Reservoir), of each submerged macrophytes. Significant differences between treatment and control are marked as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (t-test).

suppress phytoplankton (Crawford, 1979; Jasser, 1995). We will need to conduct *M. aeruginosa* growth experiments with extracts from *Chara* species to confirm our interpretation on *Chara* species.

In addition, our results show that there are considerable spatial variability in allelochemical inhibition of submerged macrophyte on *M. aeruginosa*. Although algal growth experiments with water from patches of some submerged macrophytes showed a general pattern to suppress *M. aeruginosa* growth as plant biomass increase, algal growth experiments with plant extracts showed considerable variability. For example, extracts from *P. maackianus* occurring at two sites indeed showed *M. aeruginosa* growth suppression while extracts of the same plant from the other site did not show any suppression. Similarly, extracts of *H. verticillata* from one site showed *M. aeruginosa* suppression while other extracts did not show any significant suppression. *M. spicatum* also showed various inhibiting intensity among extracts from different sites.

The observed spatial variability in submerged macrophyte suppression on *M. aeruginosa suggest* that the production of allelochemical substances may be inducible from environmental signals (Schoonhoven *et al.*, 2005). Production of allelochemical substances are generally understood as a defense mechanism of plants (Schoonhoven *et al.*, 2005). Because secondary metabolism to produce allelochemical substances would be energy-consuming process for plants or self toxic, plants are expected to produce such defense mechanism only when it is necessary (Hadacek, 2002; Schoonhoven *et al.*, 2005).

Our present study and the previous study suggest that *M. spicatum* is the best candiate to produce allelochemical substances to reduce *M. aeruginosa* growth among submerged plants examined in this study. From this, we can further proceed to identify which substances are responsible to reduce cyanobacterial growth.

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST) (No. R01-2006-000-11096-0) and Intramural Research Funds by Ajou University granted in 2005.

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(Manuscript received 31 August 2007,
Revision accepted 30 November 2007)