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Effect of encapsulated and free-living cells of *Chlorella vulgaris* L. on nitrogen retention in soils**

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Abstract. We hypothesised that the addition of free-living and alginate-encapsulated algae *Chlorella vulgaris* to the soil would change the availability of soluble forms of nitrogen, increasing the retention of nitrates, which is especially important due to fertilisers misuse and nitrogen leaching. *C. vulgaris* were grown on Knop and Baslerowa-Dvorakova media. The best growth was observed on Knop medium in 25ºC. Three different soils (Brunic Arenosol, Haplic Umbrisol, Mollic Umbrisol) were tested in both flooded conditions and conditions corresponding to field water capacity. Capsules prepared with 1.0-2.5% sodium alginate and 0.5-5% $CaCl_2$ kept shape and consistency, but at a different level of durability. From nine different concentrations of alginate used to form the capsules, 1% proved to be the most suitable. In contrast to encapsulated *C. vulgaris,* the addition of free-living algae had a positive effect on the reduction of NO_3^- in non-flooded soils, which can be beneficial in terms of reducing N leaching. Encapsulated microalgae seemed to have assimilated NH_4^+ under flooded conditions, but this effect was generally blurred by alginate capsule sorption/adsorption. In two sandy and one silty soil, encapsulated algae were rather ineffective, and their impact was limited to a minor reduction of NO_3^- and NH_4^+ content under flooded conditions.

Keywords: alginate, sodium alginate, encapsulation, *Chlorella vulgaris*, nitrogen fertiliser application, soil

INTRODUCTION

Microalgae and microalgae-based products find application in various branches of everyday life and industry worldwide (Ryan *et al.*, 2009). Microalgae have a large impact on biogeochemical cycles, *i.e.* interactions between the atmosphere, the biosphere (living organisms), the hydrosphere (water reservoirs) and the lithosphere (minerals of the Earth's crust) on Earth. They play an important role in the circulation of carbon (C), nitrogen (N), phosphorus (P), sulphur (S) and oxygen (O_2): they were proved to provide about half of the atmospheric O_2 (Brodie *et al.*, 2017). Additionally, intensive farming of microalgae can contribute to a reduction of greenhouse gases in the air: approximately 50% of dry algal biomass is carbon from the atmospheric CO_2, and it is consumed in amounts of 183 g per 100 g of microalgae biomass (Mirón *et al.*, 2003; Chisti, 2007). The production of microalgae biomass requires adequate amounts of CO_2, H_2O, light, and essential macro- and micro-elements, of which N, S and P are the most important.

Chlorella vulgaris (*C. vulgaris*), one of the most common algae, is a persistent and rapidly growing microalgae from the green algae cluster. Species belonging to the genus *Chlorella* can be found in both freshwater and marine water habitats. *Chlorella sp.* has a simple life cycle and simple nutritional requirements (Richmond, 2007).

Encapsulation is a process of closing different substances in a semi-permeable membrane capsule (Dusseault *et al.*, 2005; Vemmer and Patel, 2013). The capsule can release its contents in a controlled manner under specific conditions, *e.g.* at certain pH and gradation of compound concentrations on both sides of the membrane (Wan *et al.*, 1997; Naga Pavan Kumar *et al.*, 2012). So far, various types of polymers have been experimented with, such as: sodium alginate, agarose, polyacrylates, polyvinyl alcohol, chitosan, and poly-L-lysine, for use in the encapsulation of living cells (Thu *et al.*, 1996; Lee and Chu, 1997; Temprano *et al.*, 2002; Young *et al.*, 2006; Schoebitz *et al.*, 2013).

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Efforts were made to improve the quality of immobilisation, which would contribute to the maintenance of cell function and overall survival rate (Angelova and Hunkeler, 1999; Orive *et al.*, 2004; Vemmer and Patel, 2013). The type of encapsulation method depends on the purpose and conditions of capsule use. Therefore, a number of modifications to the classical methods of encapsulation were proposed (Schoebitz *et al.*, 2013; Vemmer and Patel, 2013).

Among the various natural polymers that can be used to prepare semi-permeable membranes, alginate is one of the most frequently used. It is due to the fact that its gelation does not require much specialised work or any special reaction conditions. It is also highly biocompatible with various materials and non-toxic (Jen *et al.*, 1996; Goh *et al.*, 2012; Schoebitz *et al.*, 2013). Alginates are linear copolymers consisting of naturally occurring β-D-mannuronate (blocks M) and α-L-guluronate (blocks G) residues. Blocks G are responsible for the gelation of alginates (Draget *et al.*, 2005; Goh *et al.*, 2012). Divalent (Ca^{2+}, Ba^{2+}, Sr^{2+}, Fe^{2+}) or trivalent (Al^{3+}) metal cations are involved in the formation of ionic bonds between the G units in the polymer chains. In this way, three-dimensional, ionotropic gels are formed (Chandy *et al.*, 1999; Banerjee *et al.*, 2007; Schoebitz *et al.*, 2013). To enable molecular transport of nutrients and survival of the cells inside the capsule, it is important that the membrane is semi-permeable (Kizilel *et al.*, 2005; Schoebitz *et al.*, 2013). Capsules should have adequate mechanical stability, and this depends on the concentration of calcium chloride and sodium alginate. The higher the concentration, the denser the polymer network of the capsule, which affects the deformation and elasticity parameters (Leick *et al.*, 2010; Soto *et al.*, 2018).

Nitrogen is one of the major nutrients needed for microalgae growth and one of the main components of amino acids, proteins, nucleic acids and chlorophyll in their cells (Scragg *et al.*, 2002; Chia *et al.*, 2013, 2015; Ikaran *et al.*, 2015). The synthesis of glutamic acid, which is the initial substrate for the chlorophylls in plastids, is particularly important (Jensen and Leister, 2014). Ikaran *et al.* (2015) reported that N depletion restricts *C. vulgaris* cell growth and results in a strong reduction of N-rich cell compounds. Nitrate (NO_3^-) and ammonium (NH_4^+) forms are most often used as sources of nitrogen (Covarrubias *et al.*, 2012; Griffiths *et al.*, 2014; Chia *et al.*, 2015), but some species of microalgae can also take up urea or amino acids (Hsieh and Wu, 2009). Iron cations – necessary for the operation of nitrate reductase – are required for the absorption of the nitrate form (Kwietniewska *et al.*, 2012; Ikaran *et al.*, 2015).

Nitrogen fertilisers, especially in excessive doses, have a detrimental effect on the environment: N_2O emission increases the greenhouse effect, and leaching NO_3^- contaminates groundwater (Di and Cameron, 2002; Machefert *et al.*, 2002). In addition, global demand for N fertilisers increased between 2013 and 2014 by 1.5%. It is expected that the demand will reach about 119 400 000 t in 2018, representing an annual growth of 1.4% (WFT, 2015). Due to the global effects of the use of nitrogen fertilisers, there is a need to develop more environmentally friendly practices without reducing crops.

All the above mentioned issues were the reason for undertaking this research. We hypothesised that adding *C. vulgaris* to the soil would reduce the nitrogen concentration in the soil solution, as this element would be embedded in the biomass of the algae. Increasing the retention of nitrates in the soil is likely to have a positive effect just after fertilisation, when the concentration of nitrogen is high. As a result, especially after heavy rains, nitrogen compounds are washed away from the arable layer. The biomass of algae would store nitrogen when its concentration is excessive, and release it slowly afterwards. The second hypothesis was that the encapsulation of the algae may help control the effect of nitrogen release process, thus providing a long-term fertilisation effect.

The aim of the study was to assess the effect of applying the free-living *Chlorella vulgaris*, in suspension and immobilised in capsules, on the contents of soluble forms of nitrogen in the soil.

MATERIALS AND METHODS

Tested materials were mineral soils: two sandy soils (Brunic Arenosol and Haplic Umbrisol) and one silty soil (Mollic Umbrisol), collected from upper layers of soil profile (to a depth of 10 cm), air-dried and sieved to 2 mm. The basic properties of investigated soils and their particle size distributions, measured by laser diffraction method according to the same procedure as in Lamorski *et al.* (2014), are shown in Table 1.

Chlorella vulgaris was selected for this experiment. Two different media were used for the cultivation of the algae: Baslerowa-Dvorakova medium (B-D) and Knop

Table 1. Basic characteristics of investigated soils

Soil	NO_3^-	NH_4^+	C org. (%)	pH_{KCl}	Result between user sizes (µm)		
	(mg kg^{-1})				Clay	Silt	Sand
					0.01-2	2-50	50-2000
Brunic Arenosol	29.63 ± 1.51	7.00 ± 0.16	0.86	6.0	0.81	7.61	91.58
Mollic Umbrisol	8.19 ± 1.64	5.95 ± 0.47	1.86	6.5	9.55	72.23	18.22
Haplic Umbrisol	15.12 ± 0.37	6.96 ± 1.88	0.60	4.5	2.37	21.65	75.98

Table 2. The composition of Knop and Baslerowa-Dvorakova media used (Kwietniewska *et al.*, 2012)

Compound	Knop	Baslerowa-Dvorakova
	($g\ dm^{-3}$)	
KNO_3	0.5	0.1
$Ca(NO_3)_2\ 4H_2O$	0.5	-
KH_2PO_4	0.2	0.01
$MgSO_4\ 7H_2O$	0.15	0.01
$FeCl_3\ 6H_2O$	0.01	0.001
H_3BO_3	0.003	-
$MnCl_2\ 4H_2O$	0.002	-
NH_4VO_3	0.0003	-
$ZnSO_4\ H_2O$	0.0002	-
$(NH_4)_6Mo_7O_{24}\ 4H_2O$	0.0001	-

medium (Table 2). The microalgae were cultivated using sterile flasks in phytotrone FD 711 DD INOX – 2 x 350 L (Biosell) with photoperiod of 16 h/8 h light/dark for 12 days and PAR 150 µmol m^{-2} s^{-1}. The culture was shaken for 15 min every hour (80 RPM). Two temperature variants were also used for each medium separately: 20 and 25ºC (± 2ºC).The value of pH was 6.75 ± 0.2 and was monitored every day (Hach Lange HQ 40d multi).

For the assessment of the microalgae growth rate, a spectrophotometric approach was used (UV-1601PC Shimadzu). The optical density (OD) of different media was measured on 680 nm every day (Dziosa and Makowska, 2016).

Capsules were made of alginic acid sodium salt form brown algae powder and calcium chloride dihydrate powder ($CaCl_2\ 2H_2O$) purchased from Sigma-Aldrich (UK).

The dripping-ionic gelation beads method was used for encapsulation (Vemmer and Patel, 2013). The process of complete dissolution of alginate occurred after several hours using a magnetic stirrer. Capsule formation consisted of instilling a viscous concentration solution of sodium alginate into concentration calcium chloride using a syringe. The formation of the gel occurs when Ca^{2+} ions react with the outer layer of alginate droplets. During this process, as a result of the reaction of the sodium salt of alginic acid and calcium cations of Ca^{2+} coming from dissociation, capsules are formed with a gel consistency. Next, the capsules were left in the solution for about 15 min to polymerise completely. After this time, the capsules were rinsed with water and re-placed in a calcium chloride solution.

Ninety variants were tested in the experiment: 9 types of different sodium alginate concentrations, ranging from 0.5 to 2.5% (increase by 0.25%) and 10 concentrations of C% Ca^{2+}, ranging from 0.5 to 5% (increase by 0.5%).

The formation of the capsules with immobilised microalgae required the adjustment of the adopted concentrations: 2% sodium alginate solution was mixed with microalgae to obtain 1% of the mixture; then, we proceeded the same way as with empty capsules – the mixture was added dropwise to 1% calcium chloride using a syringe.

Diffusion tests were performed to determine the degree of diffusion through the walls of the capsule and in order to select the optimal concentration of the compounds used to form capsules. The capsules were stained by adding methyl-red chloride to the solution of calcium chloride. After this stage, a standard capsule-forming procedure was applied. Before being measured and placed in a cuvette of water, stained capsules were cleaned using tissue paper. This treatment allowed us to eliminate measurement errors caused by water contamination with a dye on the surface of freshly prepared capsules. The absorbance changes were then monitored for approximately seven hours, *i.e*. until the values reached a constant level (UV-1601PC Shimadzu).

Soil samples (5 g dry mass) were incubated in glass bottles (20 ml) under controlled temperature (20 ± 2ºC), and N forms, nitrate (NO_3^-) and ammonium (NH_4^+) content was determined on the 1st, 3rd and 7th day of incubation. Four variants were prepared: (i) control (soil without any additions), (ii) soil with free-living microalgae addition (1 cm^3), (iii) soil with alginate capsules with immobilised microalgae (40 pcs of capsules which correspond to 1 ml of microalgae and 1 ml of alginate), (iv) soil with alginate capsules (40 pcs). The dose of encapsulated algae was large and can be used in specified conditions. The incubation soil with algae was carried out in phytotrone FD 711 DD INOX under controlled temperature (20 ± 2ºC), on the lit conditions 150 µmol m^{-2} s^{-1}.

Mineral N was determined under two moisture levels: flooded and corresponding to field water capacity (FWC) (water potential pF 2.2). Soil water contents (w/w) proper for pF 2.2 were: 10-12% for sandy soils Brunic Arenosol and Haplic Umbrisol, and 23-25% for silty soil Mollic Umbrisol. Nitrogen-form content was analysed in the 0.01 M $CaCl_2$ extracts using FIA Star 5000 auto-analyser (FOSS Tecator).

RESULTS

The growth of microalgae on different media is presented in Fig. 1. In general, *C. vulgaris* grew better in Knop medium than in B-D medium. The growth curve in B-D medium did not show the typical kinetics of normal algal growth and was very flat. The algal culture in Knop medium at 20ºC reached the stationary phase on day 8th, while at 25ºC on day 9th. OD at that stages were 0.124 and 0.166, respectively. Growth of the microalgae on Knop medium was approx. three times better than on B-D medium (after eight days of growth). The effect of higher temperature was visible on Knop medium, but it can be possibly unjustified from the economical point of view.

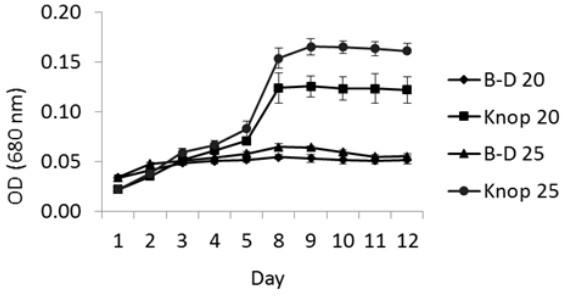


Fig. 1. Growth rate of *C. vulgaris* expressed as optical density (OD) on Baslerowa-Dworakova (B-D) medium and Knop medium at two temperatures: 20 and 25ºC.

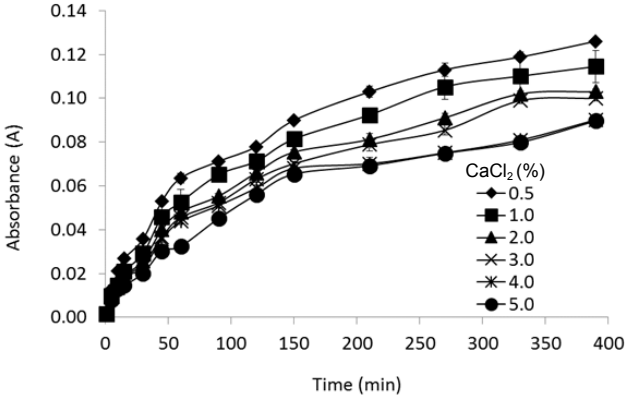


Fig. 2. Methyl red diffusion through membrane capsules according to the $CaCl_2$ concentration used.

The capsules produced with 0.5 to 0.75% sodium alginate were considered as unsuitable for further research – they were characterised by poor mechanical parameters and spontaneously disintegrated. Capsules prepared with 1.0-2.5% sodium alginate and 0.5-5% calcium chloride kept shape and consistency, but at a different level of durability. 1% sodium alginate was selected for further analysis and six levels of $CaCl_2$ were tested for methyl red diffusion: 0.5, 1.0, 2.0, 3.0, 4.0, 5.0%. Tests with red methyl showed that diffusion occured in all capsules, although at various levels. As could be predicted, as the concentration of $CaCl_2$ increased, the membrane permeability of the capsules decreased (Fig. 2).

The differences in N forms content between the experimental variants were detected on the 3rd and 7th day of incubation depending on soil type and soil water content (Fig. 3). As tested soils varied in native nitrogen content (Table 1), the differences between them were detected from the 1st incubation day. The highest NO_3^- content occurred in Brunic Arenosol (Fig. 3A), followed by Haplic Umbrisol and Mollic Umbrisol soil, while NH_4^+ content was similar in all soils at the beginning of incubation, regardless of the variant of the experiment.

In the flooded Brunic Arenosol, the decrease of NO_3^- and increase of NH_4^+ during incubation occurred in all tested variants (Fig. 3A). Nitrate content decreased significantly ($p < 0.05$) in the 3rd day of incubation in soil with free-living algae and soil with microalgae immobilised in capsules. On the same incubation day, Brunic Arenosol NH_4^+ content did not change. On the 7th day of incubation,

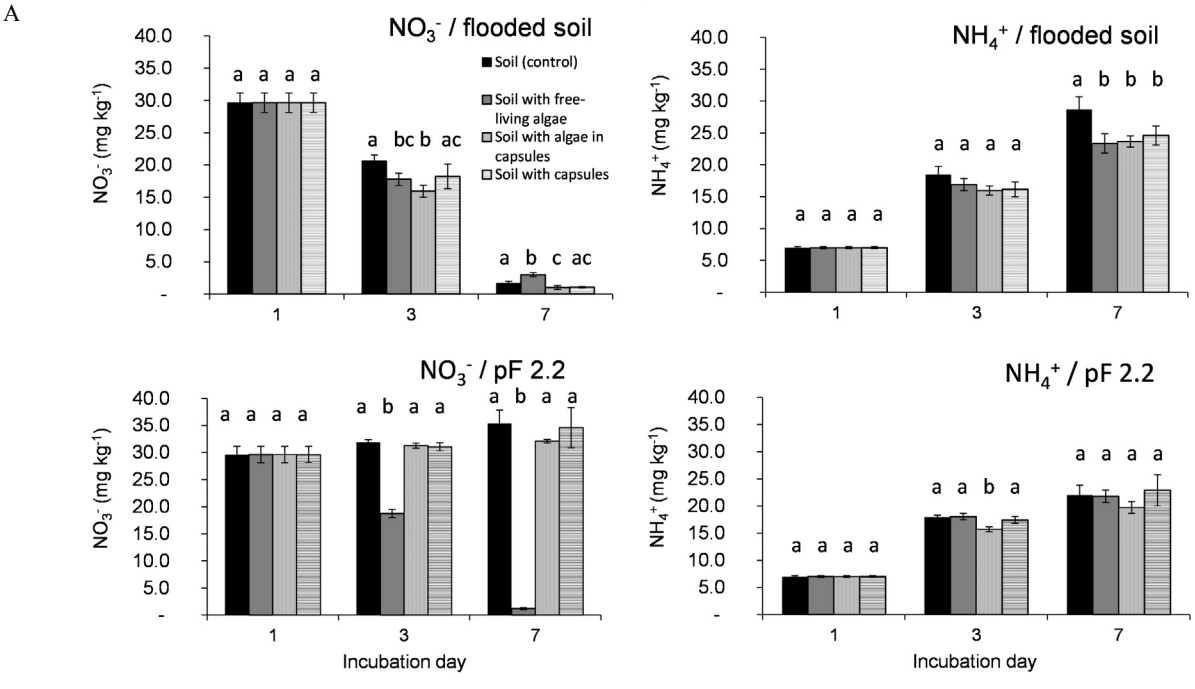
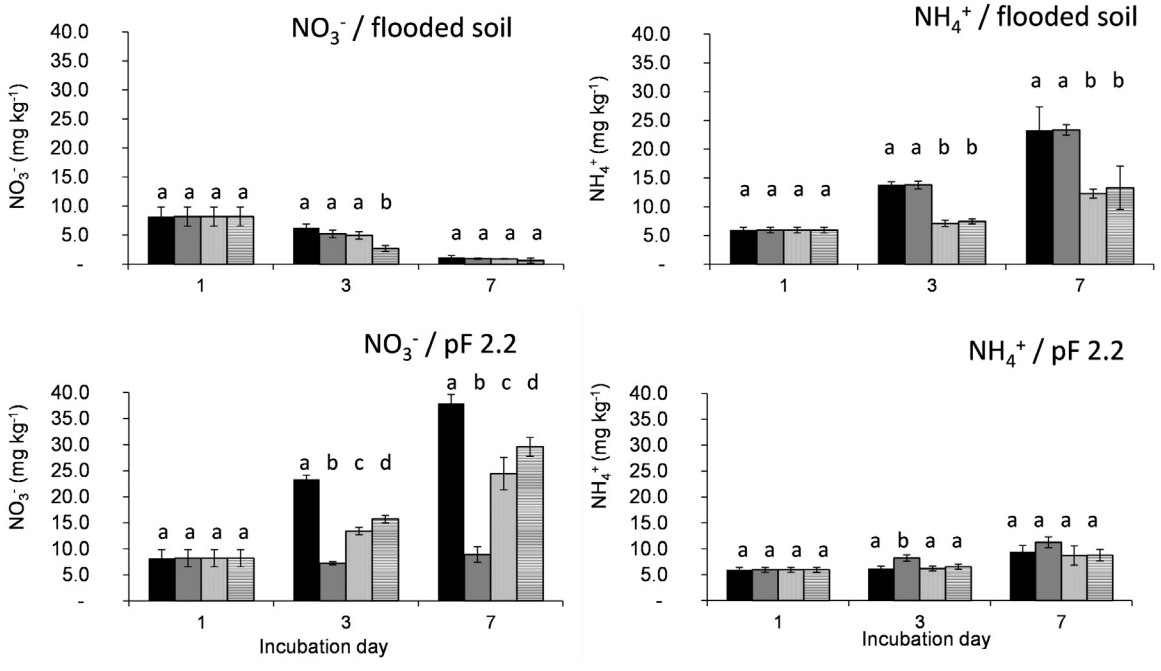


Fig. 3. Mineral N forms content (NH_4^+, NO_3^-; averages ± SD, n = 3) in soils: A – Brunic Arenosol, B – Mollic Umbrisol, C – Haplic Umbrisol; on the 1st, 3rd and 7th day of incubation under two moisture levels: flooded and corresponding to water potential pF 2.2 (field water capacity). The letters indicate a significant statistical difference; one-way ANOVA, Tukey HSD test, $p < 0.05$.

B



C

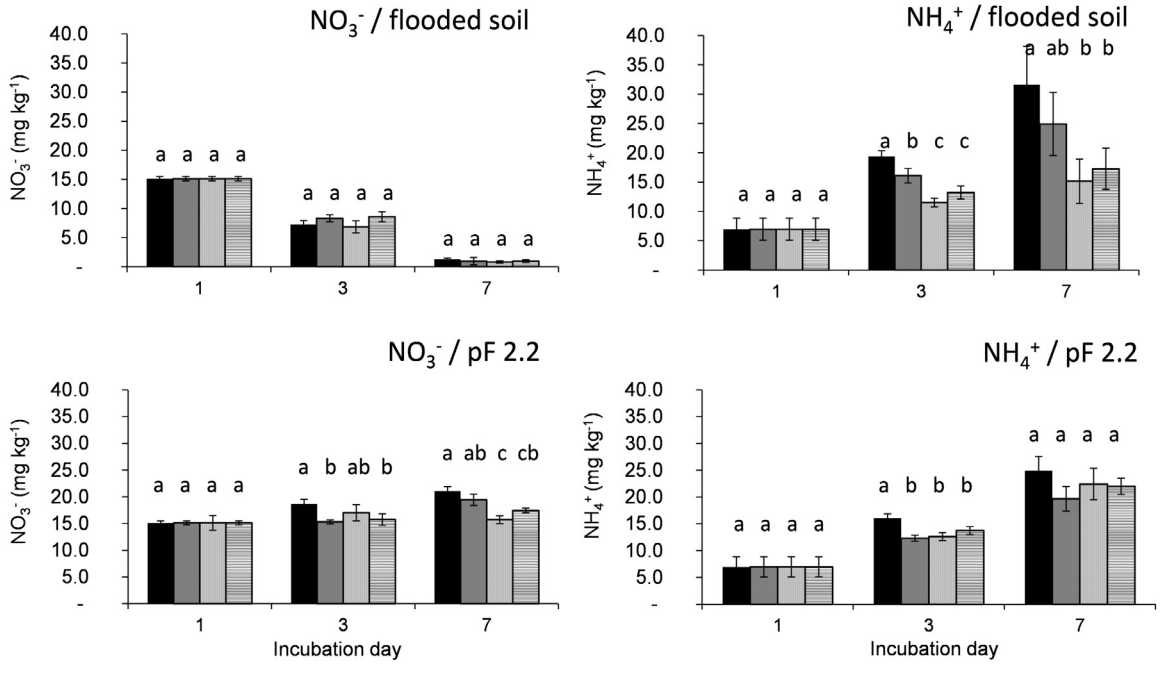


Fig. 3. Continuation.

the NO_3^- content was low (about 1-2 mg dm^{-3}) in all variants under flooding. The encapsulated microalgae decreased nitrate content while free-living algae increased NO_3^- content. NH_4^+ was the highest in control soil and lowest in soil with free and encapsulated microalgae ($p < 0.05$). Under pF 2.2, a significant decrease of NO_3^- content both on the 3rd and 7th day occurred only in soil with free-living algae. As in flooded soil, the lowest NH_4^+ content was detected in the variant with soil enriched with immobilised microalgae.

In Mollic Umbrisol, N forms content was different for two moisture levels (Fig. 3B). The general tendency under flooding was similar as in Brunic Arenosol: NO_3^- content decreased, while NH_4^+ increased during incubation time. In the 3rd day of incubation of Mollic Umbrisol, NO_3^- content decreased significantly ($p < 0.05$) only in soil with empty capsules, while NH_4^+ content decreased also in encapsulated microalgae. On the 7th day of incubation under flooding there were no significant differences

in NO_3^- content between individual variants. Different situation was found for NH_4^+ content, which significantly ($p < 0.05$) decreased in variants with encapsulated microalgae and empty capsules. Under lower moisture (pF 2.2) on the 3rd and 7th day in Mollic Umbrisol (Fig. 3B), the lowest NO_3 content occurred in soil with free-living microalgae. Content about two (3rd day) and five (7th day) times higher – but still significantly lower than control – was noticed in other two variants (encapsulated microalgae and empty capsules). On the 3rd incubation day, the highest NH_4^+ content was detected in soil with free-living algae. However, on the 7th day, capsule addition to soil did not have a significant effect on NH_4^+ content in Mollic Umbrisol under pF 2.2.

Haplic Umbrisol was characterised by nitrate content about two times lower than that of Brunic Arenosol but two times higher than that of Mollic Umbrisol (Table 1). Its concentration varied depending on the water conditions (Fig. 3C). Nitrate content in Haplic Umbrisol under flooding was similar in all variants throughout the experiment. A significant decrease in NH_4^+ content occurred in all variants (except for free-living microalgae on the 7th day) during the experiment. In Haplic Umbrisol incubated under lower moisture (pF 2.2) (Fig. 3C), NO_3^- content under pF 2.2 was rather stable during the experiment, in contrast to the two other tested soils. However, on the 3rd day, it was slightly but significantly ($p < 0.05$) mitigated in free-living microalgae and empty capsules, while on the 7th day, significant changes appeared in encapsulated microalgae and empty capsules variants. Similarly to the flooded conditions, NH_4^+ content on the 3rd day was decreased, but on the 7th day was on the same level as in all variants.

In general, the NO_3^- content in soils decreases as a function of time in flooded soils, while the NH_4^+ content increased in both flooded and moist soils. The average NO_3^- content in soils under pF 2.2 increased.

DISCUSSION

Several forms of nitrogen can be used by microalgae in the growth process: nitrate, nitrite, ammonium and some other (Becker, 2008). This can be environmentally beneficial for maintaining N in soil for a longer period of time, and lower the risk of leaching, which was the aim of this experiment.

Algal growth in Knop medium showed typical growth kinetics (Fig. 1). In B-D medium the increase of OD was minimal, indicating that this type of medium was too poor for good *C. vulgaris* growth. Wong *et al.* (2017) reported the highest OD in the Bold basal medium (3.389). However, this type of medium has a high content of inorganic salts and vitamins, and is expensive for mass microalgae breeding. Knop medium seems to be the optimal choice, because it consists of only inorganic salts. With the use of this medium, an OD of 0.124 at the beginning of stationary phase at 20ºC was shown (day 8). This was a better result than, for instance, the one obtained with LC Oligo medium at the same temperature (Chia *et al.*, 2013). Our results also showed that the use of a higher temperature of cultivation (25ºC) could be economically unjustified.

The production of durable and mechanically resistant capsules depends on many factors, such as the method used, the concentration of the reagents used, the rate of drop formation, or even the surface stress of the gelling solution *etc*. (Vemmer and Patel, 2013; Davarcı *et al.*, 2017). Capsules produced with 0.5 to 0.75% sodium alginate are spontaneously disintegrated. This is confirmed by studies on the effect of alginate concentration on capsule formation. The resistance of capsules increases with increasing sodium alginate. In relation to this phenomenon, we observed qualitatively that the capsules obtained from 0.75% w/v sodium alginate solutions were more resistant, from a mechanical point of view, than those obtained from less concentrated solutions (Blandino *et al.*, 1999). Capsules made with 1-2.5% of sodium alginate and calcium chloride with a concentration of 0.5-5%, are characterised by good mechanical parameters, but they have different properties. The literature states that as the alginate concentration increases, the diameter of the capsules and the viscosity of the solution increase. Thus, the viscosity of alginate solutions has a significant but limited impact on droplet diameter (Davarcı *et al.*, 2017). The stability of the capsule increased because the thickness of the membrane increased under a higher concentration of $CaCl_2$. This outcome can be explained by the fact that an increase in the mass of calcium ions initially contained in the core capsule will result in a larger concentration gradient between the core and the outside solution. Similar conclusions were drawn by Yamagiwa *et al.* (1992) they found that the maximum coating film thickness increased with an increase in the concentration of calcium ion and in core bead diameter.

Studies have shown that dye diffusion occurs faster when the concentrations of compounds used to produce capsules are low (in particular, the concentration of calcium chloride that fills the pores of the semi-permeable membrane). The diffusion of dye from capsules with high concentrations of alginate and calcium chloride is poor. This data are similar to Yoo *et al.* (1996), where 1.0% $CaCl_2$ solution was used for the hardening of Ca-alginate capsules and the harvested cells were mixed with a 1.0% sterile sodium alginate solution. The highest diffusion efficiency was observed during the first 45-60 min of the study. This is consistent with the literature results of Naga Pavan Kumar *et al.* (2012) who in turn observed the diffusion of amylase from the capsule. The results confirmed that about 52% of the amylase was released during the first 45 minutes of incubation. Similar results were obtained by Houria *et al.* (2012) they used 1% alginate to encapsulate probiotic and incubated simulated intestinal fluid of pH 7.5.

Considering the degree of dye diffusion and the economic viability of the capsules produced, capsules made with 1% calcium chloride and 1% sodium alginate were selected for further research. Such configuration ensures the mechanical stability of capsules, saving materials used during measurements and diffusion from the inside of capsules at a level similar to variants with a richer composition.

In our study nitrogen forms content was determined under two moisture levels: in flooded conditions and in moist soils (Brunic Arenosol, Mollic Umbrisol, Haplic Umbrisol) (Fig. 3) with water content corresponding to field water capacity (pF 2.2). In addition, the water content determines the soil oxygenation level, which is strongly related to nitrogen transformations. In oxic conditions, nitrification (oxidation of NH_4^+ to NO_3^-) occurred, while under anoxic conditions, denitrification (the sequential reduction of NO_3^-, NO_2^-, NO and N_2O to N_2) took place (Stępniewski and Stępniewska, 2009; Zhu *et al.*, 2014). Therefore, mineral nitrogen is a dynamic soil component.

Our study showed that, in a combination of moisture levels, N forms content may be also affected by different additions: free-living algae, microalgae immobilised in capsules and capsules alone. Microalgae need nitrogen as an essential compound of amino acids, proteins, nucleic acids and chlorophyll (Zhu *et al.*, 2014; Chia *et al.*, 2015; Ikaran *et al.*, 2015). They absorb N in ammonium, nitrate or urea form (Griffiths *et al.*, 2014; Hsieh and Wu, 2009). After nitrate absorption from the soil solution, the algae transform it into ammonium ion, which in turn can be secreted outside, when cells have died. As nitrogen changes the metabolism of microalgae, its limitation is the most common method of enhancing lipid content (Griffiths *et al.*, 2014; Li *et al.*, 2016).

The analysis of results presented in Fig. 3 leads to a number of general observations. First, in the case of all flooded soil samples, the denitrification process and/or the reduction of nitrate ions into ammonium are clearly visible. These phenomena dominate over other transformation processes detected in the course of the experiment, and their effect can be seen in the form of a systematic drop of nitrates and increase of ammonium compounds.

Denitrification, in general, is an anaerobic microbiological process that facilitates the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), which in turn is transformed into nitric oxide (NO), nitrous oxide (N_2O) and nitrogen (N_2). Denitrification rate, byproducts content and final products depend on many conditions, including oxygen and carbon content, nitrogen forms content, pH and soil microbiology (Bai *et al.*, 2017). The other mechanism is dissimilatory nitrate reduction to ammonium (DNRA), which transforms it into nitric oxide (NO^-), nitrous oxide (N_2O) and ammonium (NH_4^+) (Ribeiro *et al.*, 2018). These two cycles can proceed simultaneously depending on soil conditions, *e.g.* carbon content, nitrate content and pH (Giles *et al.*, 2012; Balk *et al.*, 2015). Their intensity is crucial for N availability for plants and microbes. They also play a key role in N leaching and atmospheric loss (Vernimmen *et al.*, 2007).

The effect of additions (algae and alginate), at a maximum level of moisture, on the trends in nitrate concentration is not evident, though in some cases these changes were significant. However, with the soil samples at the same moisture level, the trends in concentration of ammonium ions are visibly different between the control sample and samples with additions. This is the most noticeable in the case of Mollic Umbrisol: on the 3rd and 7th day, the ammonium ion concentrations were significantly lower in samples that contained capsules and capsules with algae. Therefore, it is highly possible that the reduction of ammonium ion concentration was not due to the activity of algae, but due to the sorption by the alginates: carboxyl groups of negative charge present on their surface with affinity to NH_4^+ (Daemi and Barikani, 2012; Juárez *et al.*, 2014). This effect can be considered beneficial, as the nitrate ions are likely to be stored in the soil. Thus, the economic effect of applying encapsulated microalgae is arguable, as the same result can be obtained by means of empty capsules. On the other hand, the immobilised algae can use the absorbed ammonium ions to build their own amino acids (Liu *et al.*, 2015), and thus keep nitrogen (N) in the soil for even longer times. Empty capsules will not have this effect. Moreover, Mollic Umbrisol is a heavier soil that binds much more water than the sandy Brunic Arenosol or Haplic Umbrisol. For this reason, it is naturally richer in micropores – and, because of that, able to store more NH_4^+ ions. Therefore, the ion storage effect was most apparent in Mollic Umblisol samples.

The effect of the type of the addition (free-living microalgae, encapsulated microalgae, empty alginate capsules) was best visible with samples not completely saturated with water. The water potential (pF) of 2.2 (16 kJ m^{-3}) corresponds to the field water capacity (FWC) that is widely accepted as the water content optimal for plant growth (Bieganowski *et al.*, 2013).

The most spectacular drop in nitrate concentration was observed in Brunic Arenosol and Mollic Umbrisol (Fig. 3A and 3B) with free-living microalgae. Adding alginate capsules (either empty or with algae) to Brunic Arensol samples had no effect on nitrate content. This may have been due to the fact that the free-living algae in this soil had a direct access to the ions, which is specific for this type of soil: bringing it to the pF of 2.2 required more water than in the case of the other soils (Table 1). Therefore, the free-living microalgae could mix with water and ions, and stay in the soil solution, but the capsules had a restricted access to it. This is confirmed by the results with flooded soil samples. As for the remaining soil types, the effect of adding capsules and encapsulated microalgae was visible – on the 3rd and 7th day – but not equal. Admittedly, there were no significant differences between soil samples with empty capsules and samples with encapsulated algae,

but a clear trend was observed in samples of pH favourable for algae growth: capsules with algae reduced nitrate ion concentration more efficiently than empty capsules. With Haplic Umbrisol, (Fig. 3C), where free-living algae had almost no effect on nitrate concentration, the difference between empty capsules and encapsulated algae was not visible.

The reason for observable reduction of nitrate concentration at pF 2.2 in Mollic Umbrisol (Fig. 3B), and its lack in Brunic Arenosol (Fig. 3A) may be the contents of native nitrogen – much higher in the latter soil. With more nitrogen present in the sample, the changes in the concentration of its compounds are likely to be less visible, as the combined effect of consuming nitrates by the algae and, if applicable, their sorption on alginates is going to be relatively smaller in proportion to the total nitrate content in the system. Another reason may be the differences in soil texture, *i.e.* the different area of contact between the soil solution and the capsule.

The lack of evident changes in nitrate and ammonium concentrations in Haplic Umbrisol samples (Fig. 3C) may be due to pH of this soil being unfavourable for algae. The pH level optimal for *Chlorella vulgaris* is claimed to be between 6.5 and 8 (Rachlin and Grosso, 1991; Wang *et al.*, 2010). The significant ($p<0.05$) drop in nitrate ion concentration in the presence of capsules can be, therefore, interpreted as a positive effect of the capsules acting as buffers between the adverse environment of too-low pH and the algae.

When analysing changes in ammonium concentration in the samples of pF 2.2 (Fig. 3), it is difficult to notice any clear trends. The addition of algae has little effect on the processes in the soils. Given that the algae are traditionally believed to prefer ammonium ions because of a better energy balance, the activity of the algae should be greater than in the presence of nitrate ions (Perez-Garcia *et al.*, 2011; Sanz-Luque *et al.*, 2015). However, the absorption of NO_3^- can be inhibited by the presence of NH_4^+. As demonstrated by Wheeler and Kokkinakis (1990) and L'Helguen *et al.* (2008), the concentration of about 0.1-0.3 μM NH_4^+ seriously reduces or even completely inhibits absorption of NO_3^- in natural systems, though the scale of this effect depends on the combination of species, NO_3^- concentration and carbon content (Glibert *et al.*, 2015).

The results presented in Fig. 3 indicate another fact: namely, that the nitrate ions concentration drop at pF 2.2 was evident with two soils that were not overly acidic (Brunic Arenosol and Mollic Umbrisol), which confirms the first hypothesis, whereas the drop in concentration was much less visible (or even absent) when immobilised microalgae were applied. This contradicts the second hypothesis, and leads to the following conclusion: if we search for a method for prolonging the effect of nitrogen fertilisation, algae immobilised in alginate capsules are not the answer.

CONCLUSIONS

The conclusions on the study on *Chlorella vulgaris* and its influence on nitrogen retention in soils are as follows:

1. Free-living algae addition has a positive impact on NO_3^- content reduction in non-flooded soils, which can be environmentally beneficial in terms of reducing N leaching.

2. The involvement of encapsulated microalgae in NO_3^- assimilation or transformation is not significant under the considered soil conditions.

3. Encapsulated microalgae seem to show the ability to assimilate NH_4^+ under flooded conditions; however, this effect is generally blurred by alginate capsule sorption/adsorption.

4. In general, encapsulated algae and capsulation seems to be rather ineffective (though noticeable) in sandy soils, and its impact is limited only to slight NO_3^- and NH_4^+ content reduction under flooded conditions.

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