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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1764710> since 2020-12-18T12:50:06Z

Published version:

DOI:10.1071/MF20241

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1 **Insights into the microbial autotrophic potential of a shallow**
2 **oligotrophic alpine pond**

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20 **Abstract**

21 Carbon dioxide fixation is one of the most important biogeochemical processes worldwide, but our current
22 understanding of the distribution of microbial autotrophy and its ecological significance in oligotrophic
23 freshwater systems, and particularly in benthic habitats, is poor and mainly limited to photoautotrophic
24 organisms. In this study, we investigated the autotrophic microbial communities inhabiting the sediments of
25 a high elevation, oligotrophic freshwater pond in the North-Western Italian Alps. The abundance and
26 distribution of three different forms of the RubisCO large-subunit gene were assessed in samples collected at
27 different depths by qPCR, and correlations with sediment geochemical properties and total bacterial
28 abundance were also examined. RubisCO forms *cbbLG*, *cbbLR* and *cbbM* were all detected, with
29 abundances of 9.13-10.90, 6.93-8.77 and 6.75-7.93 Log copies per g of dry weight, respectively. For all of
30 them interannual variability overcame depth-related variability. RubisCO genes abundance was strongly
31 correlated with total bacterial abundance, and both of them were positively correlated with Ca_2^+ and Mg_2^+
32 concentration. These observations provide some first indications on the distribution of photo- and
33 chemolithoautotrophic bacteria relying on the Calvin-Benson-Bassham (CBB) cycle for C fixation in alpine
34 pond sediments, and suggest that they may represent an important component of the total benthic microbial
35 community.

36

37 **Keywords**

38 Primary production

39 RubisCO

40 Sediments

41 qPCR

42

43

44 **Introduction**

45 Benthic habitats have been reported to play a key role in supporting primary production in lentic ecosystems.
46 In particular, their contribution tends to overcome those of pelagic habitats in shallow, oligotrophic water
47 bodies: in these systems, where oligotrophic conditions penalize phytoplankton productivity, sediments act
48 as a reservoir of nutrients, foraging benthic primary producers (Vadeboncoeur *et al.* 2003; Glud *et al.* 2009;
49 Cremona *et al.* 2016; Zhang *et al.* 2020).

50 Submerged macrophytes and macroalgae are the most evident example of benthic primary producers, and the
51 importance of their contribution in carbon, nitrogen and phosphorous immobilization has been described in
52 different aquatic ecosystems (Dodds 2003; Vesterinen *et al.* 2016; Martinsen *et al.* 2017). However, other
53 players may assume a major role in systems lacking aquatic vegetation: microalgae, diatoms, dinoflagellates
54 and cyanobacteria, but also chemoautotrophic prokaryotes. Therefore, a better understanding of microbial
55 primary production potential and dynamics is fundamental in order to develop a comprehensive view on the
56 ecology of oligotrophic freshwater systems and to predict their potential response to perturbations. This is of
57 great concern especially in fragile contexts such as alpine oligotrophic freshwater ecosystems, where the
58 effects of climate change are expected to have strong impacts, for instance in terms of hydrological regime,
59 water quality and trophic status (Beniston 2003; Slemmons *et al.* 2013; Redmond 2018).

60 A powerful tool that can be applied for the simultaneous detection and quantification of such organisms is
61 the analysis of nucleic acids targeting functional genes linked to carbon fixation processes, such as ribulose-
62 1,5 biphosphate carboxylase/oxygenase (RubisCO) large subunit genes. RubisCO is one of the key enzymes
63 in the Calvin–Benson cycle, the most widespread C fixation pathway in nature (Berg 2011). RubisCO exists
64 in different forms, evolutionarily related but differing in structure, catalytical properties and substrate
65 specificity (Tabita *et al.* 2008). The most common in Eukarya and Bacteria are form I and form II, whose
66 large subunit is encoded by *cbbL* and *cbbM* genes respectively. Form I RubisCO can be further divided in
67 two groups, based on the aminoacidic sequence of the enzyme large subunit (Watson and Tabita 1997):
68 green-like, found in green algae, plants, *Cyanobacteria*, α -, β - and γ -*Proteobacteria*, and red-like, diffused in
69 red algae and α - and β -*Proteobacteria*. Form II shows lower affinity to CO₂ and lower specificity than form
70 I, suggesting a more ancient origin, and has been detected in *Proteobacteria* and dinoflagellates (Tabita
71 1999).

72 In this study we focused on an alpine clear water, oligotrophic pond characterized by the absence of evident
73 macrophytes or macroalgal cover on sediments surface. The Col d'Olen Rock Glacier Pond is located in the
74 NW Italian Alps, at the terminus of the homonymous rock glacier, covering an area of 1,600 m² and reaching
75 a maximum depth of 3 m. The pond has been previously described in terms of hydrological dynamics
76 (Colombo *et al.* 2017; Colombo *et al.* 2018), sediments geochemistry and prokaryotic diversity (Mania *et al.*
77 2019). In particular, Mania *et al.* (2019) proposed water depth as the main driver involved in microbial
78 community shaping within the pond and reported several evidences suggesting the presence of a potential
79 isle of primary production localized in the deepest area of the pond, such as higher levels of pH, DOC, TDN
80 and NH₄⁺ and presence of higher proportions of cyanobacterial sequences in deep versus shallow samples.

The aim of this study was therefore to study the portion of microbial-driven primary production linked to the CBB cycle in an alpine, periglacial context. Our objectives were to explore the autotrophic genetic potential of sediment microbial community through the quantification of RubisCO genes, and to test the influence of water depth and sediments geochemistry on the distribution of the different RubisCO forms.

Materials and methods

A complete description of the sampling procedure is reported in Mania *et al.* 2019. Briefly, 10 cm sediment cores were aseptically collected from three sampling points in the Col d'Olen Rock Glacier Pond, located at different water depths (S1, S3 = 1 m, S2 = 3 m), for two consecutive years, during the snow-free season. At each sampling point three replicate samples were collected at a distance of approximately 50 cm. Total DNA was extracted and quantified as described in Mania *et al.* 2019. The abundance of genes encoding for RubisCO form I green-like and red-like (Paul *et al.* 2000; Selesi *et al.* 2005) and form II (Alfreider *et al.* 2003) was assessed by quantitative PCR (qPCR). qPCR reactions were performed using a Chromo4™ Real Time PCR Detection System (Bio-Rad Laboratories), in a reaction volume of 20 µl, including 10 µl of SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), 0.3 µM of each primer and 2 µl of template DNA (diluted to less than 20 ng µl⁻¹). Primer pairs and reaction conditions are summarised in Table 1. Each sample was analysed in triplicate, and product specificity was confirmed by melting curve analysis and visualisation on agarose gel. For standard curves setup PCR products were obtained from environmental samples or genomic DNA of reference organisms by applying the same cycling conditions used for qPCR with the addition of a final elongation step (Table 1). PCR products were purified with the PCR Extract Mini Kit (5 Prime), quantified by Qubit® (Life Technologies) and serially diluted in molecular grade water. The standard curves were analysed in triplicate, and reported R² values higher than 0.99 and efficiencies of 66%, 52% and 68% for *cbbL* red-like, *cbbL* green-like and *cbbM* respectively. Gene abundance was compared among different sampling points and years by using 2-way ANOVA. Pearson's correlation coefficients were calculated to highlight significant relationships between RubisCO genes abundance and other parameters previously assessed on the same sediment samples (Mania *et al.* 2019): geochemical properties; bacterial 16S rRNA genes abundance, quantified by qPCR; cyanobacterial 16S rRNA genes proportion over total bacterial sequences, determined by 16S amplicon sequencing. All the statistical analyses were performed in R, version 3.4.0 (R Core Team 2017).

Results and Discussion

The first objective of this study was to explore the potential of microbial communities in terms of C fixation in an alpine oligotrophic pond by assessing the abundance of the three most common forms of RubisCO genes. The abundance of RubisCO genes followed the order *cbbLG* > *cbbLR* > *cbbM*, ranging from 9.13 to 10.90, 6.93 to 8.77, and 6.75 to 7.93 Log copies per g of dry weight, respectively (Fig. 1).

Previous studies on markers of autotrophy have reported the prevalence of form I RubisCO among autotrophic communities in the water column of different oligotrophic aquatic ecosystems in cold areas

(Kong *et al.* 2012a; Kong *et al.* 2012b; Dolhi *et al.* 2015). Moreover, a recent survey on freshwater microbial communities in high-elevation catchments in the Tibetan Plateau (Kong *et al.* 2019) showed a prevalence of RubisCO sequences ascribable to the red-like form I over the green-like form I. In our system, the high levels of *cbbLG* genes could be connected with the presence of relevant proportions of cyanobacterial sequences described in the same samples by Mania *et al.* (2019), although a direct correlation between RubisCO genes abundance and cyanobacterial relative abundance was not found. Instead, given the low discrimination against O₂ and the poor affinity for CO₂ for form II RubisCO (Badger and Bek 2008), there is the possibility that a well-mixed and shallow pond is less favourable for the spread of microaerobic/anaerobic autotrophs.

An exact calculation of the proportion of CO₂-fixing bacteria on the total bacterial community is not achievable based on functional gene abundance data. Nevertheless, supposing that (i) the average copy number of 16S rRNA copies per genome in the bacterial cells is four to six and (ii) the average number of *cbb* operons in bacteria is two (Yuan *et al.* 2013; Lynn *et al.* 2017), then we can estimate that 2-3% of the bacteria in the pond sediments may have the potential to fix CO₂ through the CBB cycle.

Considering RubisCO genes distribution, no significant differences were reported among sediment samples collected in different areas of the pond, at different water depth (Fig. 1). This is possibly due to the limited variation in water depth, ranging from 1 to 3 m among the samples, and not apparently associated to variations in light and O₂ availability. However, significantly higher levels of all the genes were detected in 2015 (*cbbLG*: $F_{(1,18)} = 32.15$, $P < 0.001$; *cbbLR*: $F_{(1,18)} = 31.56$, $P < 0.001$; *cbbM*: $F_{(1,18)} = 23.17$, $P < 0.001$) if compared to 2016. Seasonal variations in benthic bacterial community structure and diversity have previously been shown to potentially overcome spatial variations, although information on microbial abundance is not available (Wan *et al.* 2017). In our case all the data refer to the late summer period, but it is interesting to highlight how in 2015 the early snowmelt led to a particularly prolonged snow-free season (Colombo *et al.* 2018), that might be related to the higher abundance of RubisCO and 16S rRNA bacterial genes. Indeed, analogous trends in total bacterial abundance have been described in the same samples by Mania *et al.* (2019), and the existence of a positive correlation between RubisCO and 16S rRNA genes copy number (Table 2) may indicate either that the variation in RubisCO genes abundance between 2015 and 2016 is ascribable to fluctuations in the overall bacterial population, or that autotrophic microorganisms actually represent a conspicuous component of the whole bacterial community.

Looking at the relationships existing between microbial markers and sediments geochemistry (Table 2), a significant positive correlation linked Mg²⁺ and Ca²⁺ to all the investigated RubisCO forms. This is not surprising, considering that Mg²⁺ is a fundamental cofactor involved in RubisCO catalytic activity (Andersson 2008). Moreover, Ca²⁺ availability has been shown to have an impact on cell viability, stress tolerance, maintenance of photosynthesis and RubisCO genes expression in Cyanobacteria (Tiwari *et al.* 2019). Other significantly positive correlations were found between Mg²⁺, Ca²⁺ and bacterial 16S rRNA gene abundance. As inorganic nutrients concentration may be a limiting factor for microbial communities in oligotrophic systems, also in this case we cannot clearly define which kind of relationship links total

155 bacterial abundance and the abundance of the autotrophic bacterial component.

156 Interestingly, the absence of depth-related trends in RubisCO genes abundance, particularly for the *cbbLG*
157 form, seems to be in contrast with previous evidences suggesting the occurrence of higher proportions of
158 *Cyanobacteria* in prokaryotic communities in the deepest area of the pond (Mania *et al.* 2019). The
159 impossibility of assessing quantitative variations in taxa abundance within a community by using relative
160 abundance data (Widder *et al.* 2016) could in part explain this discrepancy. However, the picture previously
161 obtained from metabarcoding data was supported by the presence of a positive correlation between
162 *Cyanobacteria* relative abundance and geochemical parameters potentially related to N fixation activity such
163 as TDN and NH_4^+ . Another potential explanation for the differences observed between relative abundance
164 data and RubisCO gene trends can be found in the composition of the cyanobacterial community. Indeed, for
165 the most abundant OTU in S2 samples an identification beyond the family level was not possible by using
166 the SILVA database (Quast *et al.* 2013), and also when compared to accessions in the NCBI database it
167 showed high sequence similarity with *Cyanobium* species but also with several uncultured *Cyanobacteria*
168 detected in benthic ecosystems (Mania *et al.* 2019). Therefore, we can hypothesize that the primers used in
169 this study, previously designed on a limited number of available RubisCO gene sequences (Paul *et al.* 2000;
170 Alfreider *et al.* 2003; Selesi *et al.* 2005), may have failed to amplify this particular variant, potentially
171 leading to a biased result in final *cbbLG* genes abundance. This is a common issue in molecular ecology
172 studies relying on PCR-based techniques (Tremblay *et al.* 2015; Fischer *et al.* 2016), that could be overcome
173 for instance by following a metagenomic approach, also suitable for the association of a predominant
174 phylotype to correspondent functional genes.

175

176 **Conclusions**

177 With this study we confirmed the genetic potential of the benthic microbial community of a shallow,
178 oligotrophic alpine pond in terms of autotrophy based on the CBB cycle. All the investigated RubisCO
179 forms, despite differing in overall abundance, showed a homogeneous distribution across the pond, not
180 influenced by variations in water depth, while a significative interannual variability was reported. The strong
181 link between RubisCO and bacterial 16S rRNA genes abundance, as well as the correlations with the same
182 geochemical properties suggest that autotrophic organisms relying on the CBB cycle for C fixation may
183 represent a relevant proportion of the total bacterial population in this kind of ecosystem.

184

185 **Conflicts of Interest**

186 The authors declare no conflicts of interest

187

188 **Acknowledgements**

189 This research did not receive any specific funding

190

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296

297 **Table 1 Primer pairs, amplification conditions and standard organisms used in this study**

Primer pair	Amplification protocols	Reference	DNA for standard preparation
<i>cbbM</i> F <i>cbbM</i> R	95 °C 4 min; 35 cycles: 95 °C 45 s, 57 °C 45 s, 72 °C 1 min, 85 °C 10 s; (72 °C 10 min) ^a	Alfreider <i>et al.</i> , 2003	<i>Thiomonas intermedia</i> DSM 18155
<i>cbbLR</i> F <i>cbbLR</i> R	95 °C 4 min; 32 cycles: 95 °C 1 min, 57 °C 1 min, 72 °C 1 min 30 s, 85 °C 10 s; (72 °C 10 min) ^a	Selesi <i>et al.</i> , 2005	Environmental isolate cultured from sample S2.3.15
<i>cbbLG</i> F <i>cbbLG</i> R	95 °C 3 min; 35 cycles: 95 °C 1 min, 52 °C 1 min, 72 °C 1 min 30 s, 85 °C 10 s; (72 °C 10 min) ^a	Paul <i>et al.</i> , 2000	Environmental sample S2.3.15

^a Final elongation was excluded from qPCR protocol and used only in PCR reaction for standard preparation.

298

299

300 **Table 2 Correlation analysis (n = 18) among RubisCO genes abundance assessed in this study and**
301 **bacterial 16S rRNA genes abundance (16S rRNA Bact), *Cyanobacteria* relative abundance (Cyano) and**
302 **geochemical properties (data from Mania et al. 2019).**

303 Pearson's correlation coefficients in bold indicate statistical significance Significance level: *P<0.05,
304 **P>0.01, ***P<0.001

	<i>cbbLG</i>	<i>cbbLR</i>	<i>cbbM</i>	16S rRNA Bact	Cyano
C/N	-0.222	-0.080	-0.261	-0.155	-0.199
DOC	0.338	0.250	-0.029	0.436	0.381
TDN	0.012	0.041	-0.238	0.133	0.560*
NH ₄ ⁺	-0.269	-0.319	-0.434	-0.169	0.669**
NO ₃ ⁻	0.213	0.078	0.300	-0.035	-0.141
pH	0.004	-0.132	-0.356	0.053	0.413
Mg ²⁺	0.576*	0.663**	0.504*	0.566*	-0.166
Ca ²⁺	0.576*	0.651**	0.447	0.573*	-0.164
K ⁺	0.246*	0.381*	0.337	0.343	-0.141
Na ⁺	0.417	0.482	0.573*	0.463	-0.027
Si	0.133	0.001	-0.086	0.162	0.321
Cl ⁻	0.232	0.382	0.553*	0.237	-0.280
PO ₄ ³⁻	-0.269	-0.266	-0.323	-0.416	-0.213
SO ₄ ²⁻	0.378	0.528*	0.483*	0.394	-0.274
<i>cbbLG</i>		0.944***	0.740***	0.898***	-0.242
<i>cbbLR</i>			0.795***	0.913***	-0.080
<i>cbbM</i>				0.680**	-0.400
16S rRNA Bact					-0.256

305

306 **Figure captions**

307 **Fig. 1** Abundance of different RubisCO large subunit genes in sediment samples collected across the Col
308 d'Olen Rock Glacier Pond. Different colours correspond to different sampling years (dark grey: 2015; light
309 grey: 2016). Each bar represents the average of three field replicates, and error bars display the standard
310 error. Different letters indicate significant differences ($P < 0.05$) assessed by 2-way ANOVA

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