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A polyphasic approach for assessing eco-system connectivity demonstrates that perturbation remodels network architecture in soil microcosms

G.P. Stamou[1], N. Monokrousos[2], D. Gwynn-Jones[3], D.E. Whitworth[3], E.M. Papatheodorou[1,4]

[1]*International Hellenic University, 14 º km Thessaloniki-N. Moudania, 57001 Thermi, Thessaloniki, Greece*

[2]*Department of Soil Science of Athens, Institute of Soil and Water Resources, Hellenic Agricultural Organization-DEMETER, 14123 Athens, Greece*

[3]*Institute of Biological Environmental and Rural Sciences, Aberystwyth University, Ceredigion, United Kingdom*

[4]*Department of Ecology, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece*

Corresponding author: E.M. Papatheodorou (ORCID-ID 0000-0003-1776-9126)

e-mail: papatheo@bio.auth.gr

Abstract

Network analysis was used to show changes in network attributes by analyzing the relations among the main soil microbial groups in a potted tomato soil inoculated with arbuscular mycorrhizal fungus, treated with low doses of *Mentha spicata* essential oil, or both, and then exposed to ten-fold higher oil addition (stress pulse). Pretreatments were chosen since they can induce changes in the composition of the microbial community. Cellular phospholipid fatty acids (PLFAs) and the activity of six soil enzymes, mainly involved in the N-cycle were measured. Networks were constructed based on correlated changes in PLFA abundances. The values of all parameters were significantly different from those of random networks indicating modular architecture. Networks ranked from the lowest to highest modularity: control, non-pretreated and stressed, inoculated and stressed, oil treated and stressed, inoculated and treated with oil and stressed. The high values of network density and $1^{st}/2^{nd}$ eigenvalues ratio are related to arylamidase activity while *N*-acetyl-glucosaminidase, acid phosphomoesterase and asparaginase activities related to high values of the clustering coefficient index. We concluded that modularity may be an efficient indicator of changes in the network of interactions among the members of the soil microbial community and the modular structure of the network may be related to the activity of specific enzymes. Communities that were stressed without a pretreatment were relatively resistant but prone to sudden transition towards instability, while oil or inoculation pretreatments gave networks which could be considered adaptable and susceptible to gradual change.

Key words: network analysis, modularity, asparaginase, glutaminase, arylamidase, clustering coefficient

1. Introduction

The global ecological system is described as a complex, self-organized, adaptive and dynamic structure being represented as a network of interactions that occur between connected nodes [1, 2]. Local networks within the ecological system can represent any biological hierarchy, such as species composition or functional groupings [3, 4]. Soil habitats may be viewed as complex subsystems within the larger ecological system [5], characterized by enhanced heterogeneity [6] and consisting of superimposed spheres such as the chemical background, the microbial community, or the enzymatic activity in soil [4].

According to Simard [5] the complexity of the soil microbial network is dynamic due to microbial adaptability because of rapidly evolving microbiota, capable of self-organization due to the existence of feedback operating across hierarchical spheres. Since soil microbial communities are in a non-equilibrium state, their composition, activity and abundance can change due to external influences most of which act as disturbance agents causing pronounced change in activity and/or abundance of biota. Existing evidence suggests that the soil microbial community responds differently to various types of disturbances because microbiota behavior varies in terms of species mortality and the development of the various microbial species [7]. In this paper we considered that disturbance caused a stress effect resulting in episodic physiological perturbation [8]. Tobor-Kaplon et al. [9] suggested that stress-induced changes in the energy budgets of soil organisms can trigger changes in ecosystem functioning, while Norris et al. [10] and Azarbad et al. [11], among many others, showed that a wide range of disturbances can exert a strong selective pressure on soil microbial assemblages. Disturbances are often episodic in natural systems. Philippot et al. [12] suggested that the response of a microbial community to stress is often dictated by prior stress exposure. Indeed an initial response to recurrent low-impact events moves the system to a new state, which modulates responses to a further more severe event [13]. Rillig et al. [13] suggest that this is due to differences in species' inherent tolerances to stress, the differential energy cost derived by the response of various strains to a pretreatment effect, and the

77 differential effectiveness of pretreatment on different organisms; therefore, the resulting
78 individual responses can likely affect composition and function of soil microbial
79 communities.

80 The relationship between the composition of the microbial community and soil
81 functioning, is a fundamental but yet unanswered question, although microbial diversity could
82 provide potential indications about such functions [14]. In order to study the magnitude of
83 relationships between microbial composition and function, most studies have addressed
84 possible links between species or phylogenetic diversity and various functions of the
85 community [15,16]. However, Prosser [17] suggested that although microbial communities
86 are highly diverse, they are also highly redundant with respect to function, so that the
87 relevance of diversity estimates to ecological functions is limited. He further highlighted the
88 value of exploring network characteristics such as connectivity, in order to explore the
89 relationship between composition and functions of the microbial community. Simard [5] and
90 Shade et al. [18] maintained that the structure of a network, in particular the values of
91 architectural properties such as connectedness, cohesion, transitivity, centrality etc. are
92 indicative of system resilience, while Sinha [19] discussed the network's architecture in terms
93 of stability-instability. Stability meant the ease of the community to return to a stable
94 condition after stress. It includes the components of resistance and resilience, i.e. the capacity
95 of a community under stress to persist and maintain (resistance) or recover their original or
96 new stable state (resilience) [11-12]. Such an architectural property is small-world topology.
97 Small world networks have global properties that suit randomness. However, at the local level
98 they resemble regular networks where the nodes form clusters of nodes that are highly
99 connected among themselves and have relatively fewer connections with the nodes of the
100 other modules [19-20]. According to Alon [21] the nodes in a module have strong interactions
101 and share common function. Another key structural property of small-world networks is the
102 existence of a large number of nodes involved in transitive triples [22].

103 Social network analysis has shown that several metrics can be used to capture the
104 architecture of complex networks. We suggest that such analyses could be used to assess the

relationships between composition and function in microbial communities. In the current study, we employed this approach to interrogate how exposure of soil microbial communities to different pretreatments and subsequent stress could affect the relationship between the architectural features of microbial networks and the activity of certain enzymes in soil. We have determined the composition of main soil microbial groups by phospholipid fatty acids analysis in a mesocosm experiment based on soil from potted tomato plants [23]. Some soils were pretreated by repeated additions of small quantities of *Mentha spicata* oil, which is toxic to some microbes and beneficial to others when used as a food source [24]. The stimulatory or inhibitory effect on microbial communities also depends on the applied dose [25] and it is not cumulative for microbial activity [26]. The essential oil is an easily degradable C source in soil. We hypothesize that the use of limited doses can cause various effects on a microbial community via competition between microorganisms for energy and nutrients. To verify this hypothesis we conducted a mesocosm experiment by inoculating tomato roots with the arbuscular mycorrhizal fungus (AMF) *Rhizophagous irregularis*, considering that AMF are involved in a plethora of synergistic or antagonistic relations with the members of the soil microbial community. In addition, AMF can affect the quality and quantity of C-rich exudates of the host plant roots thus altering the competitive conditions for energy sources [26]. Finally, following the work of Vokou & Liotiri [27], we conducted a pulse disturbance caused by the application of ten-fold exposure treatment with *M. spicata* essential oil. Considering the genetic and physiological adaptability of the microbial communities [18] it was expected that post-pulse disturbance effects would be observed on the architectural properties associated with the stability and the resilience of the network. This would be manifested indirectly in the composition of main soil microbial groups in a fashion dictated by the nature of the initial treatments. We hypothesize diversification in networks' attributes among treatments will be reflected in the activity of enzymes involved in N-cycle which is mainly microbially mediated.

2. Materials and Methods

133 **2.1. Experimental design**

134 Details concerning the experimental design and soil sampling are shown by Stamou et al.
135 [23]. A summary of the experimental design is highlighted in Fig. 1. Briefly, tomato seedlings
136 (*Solanum lycopersicun*) originating from sterilized seeds were grown in hydroponic cultures
137 for a period of three weeks and were singly transplanted into 30 experimental pots. Pots were
138 surface sterilized (2 L volume) and filled with sterilized soil-sand mixture (1500 g in each pot
139 at a soil:sand ratio 1:1 w/w). To eradicate indigenous AMF and other soil borne biota, the
140 soil-sand mixture was sterilized by autoclaving (4 h at 120 °C). Soil was an acid (pH 5) sandy
141 loam. Concentrations of organic carbon and other nutrients were as follows: C% 1.62, N%
142 0.096, P_{extr} 2.1 mg/100 g, K 1.62 mg/Kg, Ca 1.17 mg/Kg, Mg 0.18 mg/Kg and Na 0.136
143 mg/Kg. Before transplantation of seedlings into pots, the roots of 12 plants were inoculated
144 with *R. irregularis* inoculum ('inoculated pots'), while the remaining 18 were not ('non-
145 inoculated pots'). The inoculum consisting of spores and hyphal fragments of *R. irregularis*,
146 was provided by the Energy and Resource Institute, India and its concentration was 1000
147 propagules per gram. Ten days after root inoculation, we reintroduced into all 30 pots the
148 original bacterial community of the soil, by adding a bacterial suspension prepared from the
149 initially collected soil. For the preparation of the bacterial suspension, 10 g of the initially
150 collected soil were mixed with 50 ml of deionized water, the soil suspension was filtered
151 through a 21 μm sieve, and 10 ml of it was added to each pot near the rhizosphere zone.

152 Half of the AMF inoculated pots (six) along with the six non-inoculated pots were
153 treated with *M. spicata* (spearmint) essential oil. The spearmint oil was supplied by Etherio,
154 Research and Commerce, Eratera, Greece and it was pure essential oil produced after
155 distillation of *M. spicata* plants. The oil was added at a weekly rate of 1.33 ml per pot, for a
156 period of one month. The major compounds of *M. spicata* oil were carvone 63.9% and
157 limonene 13.3% followed by 1,8-cineole, β-pinene, myrcene and α-pinene in percentages 7.1,
158 2.8, 2.4 and 1.4%, respectively [23]. The experiment was conducted in a glasshouse under
159 natural light conditions for a two-month period (from mid-June to mid-August). During the
160 experiment, the day and night temperature ranged from 28 to 37 °C and 20 to 27 °C,

161 respectively. The plants were watered daily in order to achieve 60% of the water holding soil
162 capacity. No further fertilizers were added to the pots.
163 The experiment involved soil from replicated tomato pots being subjected to four different
164 treatments: pretreated by adding small quantities (1.33 ml) of *Mentha spicata*'s oil each week
165 for a period of one month; inoculated two months previously with the Arbuscular Mycorrhizal
166 Fungus (AMF; *Rhizophagous irregularis*); subjected to both treatments; untreated.
167 One month after the repeated application of oil and two months post AMF
168 inoculation, a disturbance (pulse type pressure; [18]) was exerted on soils. The disturbance
169 consisted of a ten-fold higher exposure in the amount of *M. spicata* essential oil. The
170 experimental design was fully factorial with AMF inoculation and oil addition being the
171 independent variables each with two levels (Yes-No). To monitor the effect of disturbance
172 *per se*, six non-inoculated and non-pretreated with oil soils were exposed to the higher
173 amount of oil. The concentration of individual PLFAs and the activity of specific soil
174 enzymes were the dependent variables. Two destructive samplings were undertaken at 3 and 7
175 days post pulse disturbance involving three replicates sampled on each occasion. A two-way
176 ANOVA and a two-way ANOSIM showed no quantitative and compositional temporal
177 differences in the PLFA recordings and enzyme activity in the same treatment. Hence, the
178 outputs of the day 3 and day 7 replicates per treatment were grouped and subjected to network
179 analysis.
180 Overall, there were five treatments with six replicates per treatment, giving a total of
181 30 pots in a randomized factorial design: (i) inoculated-pretreated with oil-stressed pots
182 (+AMF+Oil+Str), (ii) inoculated-non pretreated with oil- stressed pots (+AMF-Oil+Str), (iii)
183 non-inoculated-pretreated with oil- stressed (-AMF+Oil+Str), (iv) non-inoculated-non
184 pretreated with oil- stressed pots (-AMF-Oil+Str), (v) control (-AMF-Oil-Str). The design
185 allowed assessment of the independent and joint effect of the experimental interventions on
186 network metrics pertaining to interactions among the members of the microbial community.
187
188 **2.2 Enzyme activity assays**

The activities of six soil enzymes were studied. These were *N*-acetyl-glucosaminidase, acid phosphomonoesterase, urease, asparaginase, glutaminase and arylamidase. *N*-acetyl-glucosaminidase (NAG) and acid phosphomonoesterase activities were determined according to Allison and Jastrow [28], as these were modified in order to be applicable for 96-well microplates. The activity of the two enzymes is presented in units of μmol pNP g^{-1} h^{-1}. Urease activity was determined according to Sinsabaugh et al. [29]. It was expressed as micromoles of ammonium released per hour per g of soil (μmol NH_4^+ g^{-1} h^{-1}). Activities of asparaginase and glutaminase were determined according to Tabatabai [30] with enzyme activity here expressed as mg NH_4^+ $.kg^{-1}.2h^{-1}$. Arylamidase activity was evaluated according to Acosta-Martínez and Tabatabai [31]. Activity was expressed as mg *β*-naphthylamide. $kg^{-1}.h^{-1}$. All activities were expressed per unit of dry soil.

2.3 Phospholipid fatty acid analysis

Extraction and analysis of phospholipids from soil samples was always performed within a week. Briefly, this involved extraction of lipids, separation of phospholipids by column chromatography then methylation of esterified fatty acids in the phospholipid fraction. Chromatographic separation and identification of the sample components was achieved using a Trace GC Ultra gas chromatograph (Thermo Finnigan, San Jose, CA) coupled with a Trace ISQ mass spectrometry detector, a split-splitless injector, and an Xcalibur MS platform [23].

The total amount of PLFAs represented the total microbial biomass. The fatty acid nomenclature was according to Papadopoulou et al. [32]. Overall, 21 fatty acid methyl esters were identified and considered for further analysis, including the internal standard 19:0;The i-15:0, a15:0, 15:0, i16:0, i17:0 fatty acids were indicators of Gram-positive (Gr^+) bacteria [33,34,35], the 16:1ω9c was a Gram-negative (Gr^-) bacteria indicator [35], the 16:0, 17:0 were bacteria indicators in general [36] while the 10Me16:0, 10Me17:0, 10Me18:0 fatty acids were indicators of actinomycetes [37,38]. The sum of these indicators was used to calculate the bacterial biomass. The 18:1ω9c and 18:2ω9,12 fatty acids were indicators of fungal biomass [35,36], while the fatty acids 17:1 and 14:0 were mainly of microbial origin. Finally,

217 the PLFAs 18:0, 20:0, 22:0, 23:0, 24:0 were considered as indicators of microeukaryotes
218 (algae, protozoa, nematodes; [39]).
219
220 **2.4 Data analysis**
221 Network analysis is widely used for studying patterns of ties among nodes [40]. In this study,
222 the nodes stand for concentrations of PLFAs and the ties for the significant correlation
223 coefficients among individual PLFAs. First we estimated the significant correlations ($p<0.05$)
224 among the individual PLFAs i and j and then 5 correlation matrices with elements r_{ij}, one for
225 each group of pots that experienced the same manipulation, were built. The values r_{ij} were
226 equal either to the correlation coefficients among variables, whenever these were significant
227 ($p < 0.05$), or zero if they were not. Also the binary version of the correlation matrices was
228 elaborated setting the elements r_{ij} either to 0, if the corresponding nodes were not correlated
229 significantly or otherwise to 1.
230 In addition, to test the statistical significance of the network indices, 6 replicate
231 random networks with properties similar to those of the experimental networks, that is, with
232 the same network size and values either greater than the correlation threshold (0.81 for N=6)
233 or, otherwise, zero, were created employing a uniform generating function. Then, the indices
234 provided by the experimental networks were tested against the corresponding indices from the
235 random networks by using the Student t test, for N=6.
236 Each matrix was analyzed by the network analysis software UCINET 6 [41]. The
237 analysis estimated parameters pertaining to the architecture of the network (Table 1) and
238 yielded graphs where the nodes were depicted on a circular layout ordered by class of
239 structural equivalence [42].
240 Analysis was conducted in three steps: First we estimated parameters relative to the
241 network cohesion (density, compactness, shortest path, nulls; [43, 44], we next partitioned
242 nodes into groups bearing ecological meaning (classes of structural equivalence) and
243 estimated parameters referring to modularity (E-I index and transitivity) and finally we
244 explored patterns and embeddedness of each node within the setting of its connections

(centrality measures). In brief, cohesion metrics measure the connectedness of a network [44], modularity assesses the tendency of nodes to form distinct classes [45], while centrality accounts for the extent to which a given node occupies a more influential position than another [46]. A short description of the estimated metrics is given in Table 1.

In particular, the modularity of a network was assessed by considering the values of the shortest path (a value approximating the Log of the number of nodes is indicative of modularity; [47], the structural holes, the transitivity indices (a value higher than 0.40 indicates modularity; [46]) and the clustering coefficient. In addition, we examined whether our networks fall into the class of small-world networks. Actually, we estimated the 'Small-World-Ness' index S^{Δ} proposed by Humphries & Gurney [48]. A real-world network G is termed small-world if the shortest path (L_g) estimated for the network G is more or less equal to the shortest path (L_{rand}) estimated for an equivalent random graph ($L_g \approx L_{rand}$) and the clustering coefficient for G (C^{Δ}_g) is higher than that for an equivalent random graph ($C^{\Delta}_g >> C^{\Delta}_{rand}$), i.e. if $S^{\Delta}=(C^{\Delta}_g/C^{\Delta}_{rand})/(L_g/L_{rand})> 1$

In this study we identified classes of structurally equivalent nodes. Two nodes of a class were considered equivalent if they have to some extent similar relationships with other nodes in the network. To assess the degree of structural equivalence we used the R-squared correlation coefficient. The R-squared value accounts for the correlation between the partitioned data matrix and an ideal matrix with the same dimension. Each cell in the cluster of the ideal matrix is set to the average value of the corresponding cluster in the data matrix.

Among the centrality metrics in this paper we employed eigencentrality which measures how well connected a node is and also how many links its connections have. This identifies nodes with influence over the whole network.

Finally, to explore possible associations of the network architectural attributes with enzymatic activity, a Principal Component Analysis (PCA) was performed and the results were loaded onto the first axis of the corresponding biplot. The variables were assigned to clusters by applying a K-means cluster analysis (PCA and K-means analyses were conducted with Statistica7).

3. Results

Networks of correlated changes in PLFA abundance were created for each experimental treatment, and their metrics are presented in Table 2. The values of all network parameters were significantly different from those of the corresponding random networks indicating that the microbial guilds in the studied communities showed small-world characteristics (in all cases $S^{\Delta}>1$). For assessment of the stress effect, we compared data of non-pretreated and non-stressed (-AMF-Oil-Str) with stressed but non-pretreated pots (-AMF-Oil+Str). Stress initiated a minor decrease in the network cohesion, as indicated by the decreasing values of density and compactness. There was also an increase in the number of structural holes and the length of the shortest path. However, the influence of the stress remained evenly distributed among nodes, as indicated by the low eigenvector centralities estimated for both networks. Values higher than 2 were estimated for the ratio $1^{st}/ 2^{nd}$ eigenvalues and suggested that global features characterized the architecture of the network. The contribution of local configurations was negligible. Clearly, stress led to looser relationships (Fig. 2a, b).

In the controls (-AMF-Oil-Str), estimated transitivity values were higher than 0.4, while the average length of the shortest path approximated the Log of the number of nodes (Log21=1.322) and $S^{\Delta}>1$. This suggested modularity and small world properties, a trend that holds also after the stress (-AMF-Oil+Str). In both networks the nodes were partitioned into four classes of structural equivalence (R-square equals 0.788 and 0.513 respectively, $P<0.001$). At the whole network scale, positive values of the index E-I were estimated by taking into account the separation of nodes into clusters of structural equivalence. This highlighted the superiority of ties between (red coloured lines) versus ties within sub-clusters (blue coloured lines). Three main classes of structural equivalence were apparent in the -AMF-Oil-Str network. The first (red circles, density 0.98) contained only markers indicative of bacteria (five Gr^{+}, two actinomycetes and one Gr^{-}). Eight members of the first class occupy the most centralized positions in the network. The second class (blue circles, density 0.92)

included four eukaryote and one Gr^- markers, while the third class (green circles, density 0.95) was not as homogeneous as the first and the second one and included two Gr^+, one actinomycetes and two fungal markers.

Again, in the network -AMF-Oil+Str three main classes of structural equivalence were identified, but their composition was less homogeneous than in the previous network. The first class (red circles, density 0.99) included four markers for bacteria (three Gr^+ and one Gr^-) and 2 markers representing eukaryotes. In the second class (blue circles, density 0.35) there were markers for every microbial guild whereas the third class (green circles, density 0.96) included two Gr^+, one fungal and one eukaryote markers. Three Gr^+, one Gr^- and three eukaryote markers occupied the more centralized posistions.

Stressed pots, which had been pretreated only with either essential oil (-AMF+Oil+Str) or inoculated with AMF (+AMF-Oil+Str), gave networks with intermediate values for all network attributes, suggesting that both treatments resulted in less compact networks relative to those of control pot (Fig. 3a, b). It is noteworthy that the differences were more pronounced in samples pretreated with essential oil. For both networks, the estimated values for transitivity were greater than 0.4, while the values corresponding to the average length of the shortest path did not deviate from the Logs of the number of nodes and $S^A>1$. These findings imply the existence of modularity and small world properties. Nodes in these two networks were partitioned into four classes of structural equivalence. Judging from the low E-I values estimated mainly in the -AMF+Oil+Str treatment and to a lesser extent in the +AMF-Oil+Str treatment, we inferred an increased proportion of ties within sub-clusters relative to control, suggesting enhanced modularity. Again relative to the control, networks exhibited increased centralization, with values estimated for the eigenvector centralities ranging from 12 to 23%. The most centralized network was the network from the pretreatment with oil and then soil from stessed treatment. The difference in the values of the ratio 1^{st} / 2^{nd} eigenvalues should be highlighted here. For inoculated pots (+AMF-Oil+Str) the value of 2.94 indicates primacy of nodes and ties at the entire network scale over local

configurations. By contrast, in the oil treated samples (-AMF+Oil+Str), a value less than 2 showed almost equal contribution of global and local configurations.

Three main classes of structural equivalence were dististinguished in the -AMF+Oil+Str network. The first (red circles, density 0.95) included two Gr^+, one fungal and one eukaryotic markers. In the second class (blue circles, density 0.98), markers representing eukaryotes dominated, with one for fungi and one for actinomycetes. In the third class (green circles, density 0.72), markers representing bacteria (four Gr^+, two Gr^-, one actinomycetes) dominated, with one fungal marker. The most centralized positions were occupied by four eukaryotic, three Gr^+ and one fungal markers.

Four main classes were distinguished in the network +AMF-Oil+Str with density ranging from 0.87 to 0.98. The first class (red circles) was dominated by the four markers representing eukaryotes, while one Gr^+ and one fungal marker were also included. In the second class (blue circles) there were four Gr^+, along with one actinomycete and one fungal markers. In the third class, there were two Gr^+, one actinomycete and one eukaryote markers. Finally, the fourth class comprised one Gr^+ and two Gr^- markers. Two Gr^- markers followed by four Gr^+ markers and one actinomycete marker were in the more centralized positions.

In pots inoculated with AMF, pretreated with oil, and then severely disturbed (+AMF+Oil+Str), the lowest recordings for density, transitivity, and compactness were observed. We also recorded the highest values for the average length of the shortest path, and the highest percentage of missing ties (nulls). This highlights the existence of a loose network (Fig. 4). The E-I value estimated above showed almost equal participation of the ties within and between sub-clusters. This network exhibited by far the highest percentage eigenvector centralization. This indicates that the structure of the whole network was based around a few influential biomarkers, while the ratio of the 1^{st} / 2^{nd} eigenvalues revealed enhanced contribution of the local configurations of the nodes and ties to the appearance of the network. Only two main classes of structural equivalence were in the+AMF+Oil+Str netwotk. In the first (red circles, density 0.90) there were four bacterial markers (two Gr^+, one Gr^- and one actinomycete) along with one fungal marker. The second loose class (blue circles, density

355 0.29) contained only bacterial markers (three Gr^+ and one Gr^-), while the markers for
356 eukaryotes occupied marginal positions (green and mangenta colours).

357 Fig. 5 depicts the relationships between the activities of certain enzymes and metrics
358 referring to the architecture of networks, as resulted from employing a PCA model. For the
359 sake of simplicity, transitivity and compactness (which lay close to density) were omitted.
360 The first two components were highly significant and accounted for 75.5 % of the variability
361 in the data. Estimations of density and the ratio of 1^{st} / 2^{nd} eigenvalues, whose high values
362 pertained to more compact networks, were loaded close to the activity of arylamidase towards
363 the highest values of the first axis. In contrast, metrics whose high values indicated less
364 compact networks were loaded in association with the activity of urease, glutaminase, acid
365 phosphomonoesterase, N-acetyl-glucosaminidase and asparaginase, towards the lowest values
366 of the first axis. Specifically, the activities of N-acetyl-glucosaminidase, acid
367 phosphomonoesterase and asparaginase were loaded close to the clustering coefficient metric
368 I-E.

369

370 **4. Discussion**

371 In this study we analyzed data from an experiment studying the effect of an impacting
372 stress of essential oil on the composition of main microbial groups and enzyme
373 activities of a tomato pot soil either previously inoculated with AMF, repeatedly
374 exposed to low doses of essential oil, both, or neither. Compared to control pots, the
375 greatest effect on network structure was observed when pots were exposed to both
376 pretreatments prior to the stress. Azarbad et al. [11] reviewed the effects of secondary
377 stresses on already stressed microbial communities from long-term metal polluted
378 soils (‘stress-on-stress’ experiments) by using DNA based techniques. According to
379 these authors, two types of responses of primed microbial communities to secondary
380 stress were reported; (A) tolerant communities that manage to recover due to their

physiological adaptation and B) sensitive communities that are further degraded after their exposure to secondary stress. They concluded that the reaction of communities to a secondary stress depends greatly on the nature of the stress. To an extent the effects recorded in our pre-treated samples are partially in line with the type B proposed by Azarbad et al. [11]. In fact, the outcome of the priming either with AMF inoculation or by applying essential oil was the decrease of network density, compactness and centralization compared to the control, although of different extent. These downgrading processes were burdened with stress, ultimately resulting to relative fragmentation of the networks and increased importance of locality. Such architecture pertains to less resistance networks, susceptible to further degradation. The disruptive effect of the stress on the network of interactions among the members of the microbial community was more pronounced in soil pretreated with the same stressor (-AMF+Oil+Str) compared to inoculated counterparts (+AMF-Oil+Str). Thus, one of our hypotheses was proven correct; the nature of the pretreatment of a microbial community affects the response of network architecture to a subsequent stress. This further agrees with Philippot et al. [12], who discussed the behavior of the composition of microbial communities severely stressed with heavy metals and with Tobor-Kaplon et al. [49,50], who discussed disturbances by heavy metal addition or by changing heat and water regimes. However, although we share common conclusions with many researches, the use of network metrics to visualize the response of the composition of main soil microbial groups to primary or secondary stresses is novel.

Overall, networks showed structural similarity regardless of treatment, with the exception of the inoculated, pretreated with oil and stressed pots (+AMF+Oil+Str). Estimates of the average density indicated dominance of direct versus indirect ties among nodes and such enhanced values of network connectivity pointed to small-world properties and modularity. The modularity assertion is reinforced by the high values of transitivity ranging

between 0.915 and 0.742 (all higher than the critical value 0.40; [46]), by the average values of the shortest path being approximated by the Log of the number of nodes (Log 21=1.322; [47] and the values of the index S^{Δ}. The modular structuring of networks may suggest robustness in a context of environmental constraints and deprived resources [47, 51]. Modularity fitted closely at the level of structural equivalence. Consequently, in each network, the members of each class of structural equivalence would be ecologically homologous with respect to the exploitation of resources [52] and the response to environmental constraints [22, 53-55]. This was due to the fact that PLFAs provide strain profiles rather than being indicative of individual strains. Because of the coarse-scale analysis of this study, strict taxonomic or functional correspondence with the structurally equivalent groups was not possible. However, it was possible to loosely associate classes of structural equivalence with certain guilds. There was a tendency for eukaryotic markers to exhibit higher values of eigenvalues indicating enhanced centrality in stressed but non-pretreated pots (-AMF-Oil+Str), while in inoculated and stressed (+AMF-Oil+Str), as well as in control pots (-AMF-Oil-Str) more central positions tended to be occupied by markers indicating bacteria. Only three out of the seven markers indicate that Gr^{+} bacteria were positioned in the same cluster together with markers for eukaryotes.

The E-I values estimated after the partition of nodes to structural equivalence classes were positive for all networks. Positive E-I values indicated a prevalence of ties between classes and enhanced robustness of the global architecture. However, in soil pretreated with essential oil the E-I values were lowest, indicating enhanced within-class ties and increased robustness of local configurations. Results show that under pretreatment of soil with *M. spicata* oil and application of a stress of the same nature, the robustness of the local versus the global architecture of the microbial network is improved. The inter-network differences in modularity and the contribution of local configurations of nodes and ties to the global architecture of the networks were likely due to fluctuations in strains' evenness. This is because different treatments favoured different microbial groups at the expense of others. As

434 shown by Stamou et al. [23], AMF and essential oils act selectively on the main microbial
435 groups, but in divergent directions.

436 Despite evident modularity, the eigenvector centralities of the networks varied
437 between low and moderate levels, while the ratio of 1^{st} / 2^{nd} eigenvalues suggested dominance
438 of the global as opposed to the local configuration (except for the +AMF+Oil+Str network)
439 and an absence of focal nodes operating as highly influential hubs [3,56,57]. According to
440 Scott [58] such decentralized networks, where most nodes are more or less equally influential
441 and the links among them are evenly distributed, are considered more resilient. More
442 specifically, Scheffer et al. [59] suggested that more homogeneous networks are resistant to
443 change but prone to critical transition. In contrast, networks exhibiting modularity
444 accompanied by enhanced heterogeneity have increased adaptive capacity and are prone to
445 gradual change. Sinha [19] claimed that changes in the connection topology from regular to
446 random networks, does not affect the stability of a network. However, it affects the type of
447 transition in that it gets sharper as the network becomes more random. Taking into
448 consideration the E-I values and metrics relating to centrality we conclude that the networks
449 in the non-pretreated pots (-AMF-Oil-Str and –AMF-Oil+Str) were expected to be generally
450 more resistant, but susceptible to sudden transition towards instability in the sense of Sinha
451 [19]. The counterparts receiving single pretreatments were expected to have an increased
452 adaptive potential and inclination for gradual change. All metrics recorded in the
453 +AMF+Oil+Str pots indicated a highly heterogeneous network with restricted connectivity,
454 increased centralization, superiority of less influential nodes and minimal modularity,
455 therefore at risk of further disintegration. Certain biomarkers, such as 16:1ω9, 15:0, 14:0 are
456 key for the integrity of this network. The joint effect of the two pretreatment agents,
457 inoculation with AMF and repeated addition of essential oil, affected the architecture of the
458 interaction network more severely than the effects exerted by each agent alone. Probably, as
459 already hypothesized by Stamou et al. [23], without changing the total microbial biomass the
460 two preliminary treatments caused a divergence in the composition of main soil microbial

groups. Both act as selective factors though independently of each other, but each one favors and selects different microbial groups, eventually resulting in a fragmented network.

Network architectures, as associated with the activity of certain enzymes, suggested effects of the applied treatments on functional properties of the soil. There has only been one study considering PLFAs and enzymes as nodes of a network relating to the unfolding of a secondary succession process and associating individual PLFA markers, utilization of Biolog substrates and nutrients with some enzyme activities [4]. It was reported that the architectural elements of the interaction networks could be related to the activity of N-acetyl-glycosaminidase, acid phosphomoesterase and asparaginase, which appear related to the modularity of the network. Arylamidase activity was instead associated with a more coherent microbial network. It is possible that the activity of the N-acetyl-glycosaminidase, acid phosphomonoesterase and asparaginase is a consequence of cooperation among ecologically equivalent groups, while the production of arylamidase seems instead to be dependent on specific microbial strains rather than on specific microbial groups.

In conclusion, this novel use of network analysis provides insights into topological structures associated with changes in ecologically equivalent modules during the response of the main soil microbial groups to perturbation. Moreover, this approach allowed comparison of global and local attributes of the networks (glocal approach) and provided data regarding the relationships between composition and function of main soil microbial groups. In addition to deepening our knowledge on network analysis methodology, our results may have relevance to real world cultivation practices since tomato is among the most propagated crop plants.

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Conflict of Interest: The authors declare that they have no conflict of interest

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666

Figures' Legends

Figure 1. Schematic illustration of the experimental design. (each treatment has 6 replicates)

Figure2 (a, b). Projection of the network of correlations among microbial PLFAs on a circle-type plot in control (a: -AMF-Oil-Str) and only stressed pots (b: -AMF-Oil+Str). The colour of nodes corresponds to different classes of structural equivalence. The size of nodes accounts for their eigenvector centrality, specifically, the larger the node, the higher the eigenvector centrality, and the higher the influence of the corresponding node. The red coloured ties represent links between classes of structural equivalence and blue coloured ties correspond to links within classes of structural equivalence. There were no significant correlations of marker 10Me18:0 with any other marker.

Figure 3 (a,b). Projection of the network of correlations among microbial PLFAs in oil treated and stressed pots (a: -AMF+Oil+Str) and inoculated stressed pots (b: +AMF-Oil+Str). The colour of nodes corresponds to different classes of structural equivalence. The size of nodes accounts for their eigenvector centrality, specifically, the larger the node, the higher the eigenvector centrality, and the higher the influence of the corresponding node. The red coloured ties represent links between classes of structural equivalence and blue coloured ties correspond to links within classes of structural equivalence. There were no significant correlations of the outside the network marker 10Me17:0 with any other marker.

Figure 4. Projection of the network of correlations (+AMF+Oil+Str) among microbial PLFAs on a circle-type plot. The colour of nodes corresponds to different classes of structural equivalence. The size of nodes accounts for their eigenvector centrality, specifically, the larger the node, the higher the eigenvector centrality, and the higher the influence of the corresponding node. The red coloured ties represent links between classes of structural equivalence and blue coloured ties correspond to links within classes of structural

694 equivalence. There were no significant correlations of the outside the network marker
695 10Me17:0 and a17:0 with any other marker.
696
697 **Figure 5.** Ordination of parameters accounting for the architecture of the network (in green)
698 and activity of certain enzymes (in red) on a PCA biplot.
699

700 Table 1. Network properties and metrics referring to the architecture of the whole network

701

Cohesion: Assesses the extent of connectedness of a network	Density	The number of ties divided by the maximum number of possible ties
	Length of the shortest path	The minimum number of steps connecting a node with another
	Compactness	The average value of all the reciprocal distances among nodes, Accounts for the probability of two nodes to be directly tied
	Structural holes (nulls)	The number of missing ties. Accounts for the missing ties among nodes
Modularity: Assesses the possibility of various nodes to be grouped together	E-I index	The number of ties external to the cluster minus the number of ties that are internal to cluster divided by the total number of ties
	Transitivity	The number of transitive triples in a network divided by the number of the transitive and non-transitive triples
	Clustering coefficient	The clustering coefficient C is a measure of how much neighbors of each node are also neighbors of each other
	Small-worldness-index S^{Δ}	A real-world network G is termed small-world if the values of the shortest path and the clustering coefficient estimate for the network G are equal and higher respectively than the corresponding values estimated for a random graph
Centrality: Assesses the extent to which the overall network structure is dominated by one or few nodes	Eigenvector centrality	Connections to nodes with higher number of connections contribute more to the score of the ego node than equal number of connections but to nodes with lower number of connections
	Ratio $1^{st}/2^{nd}$ eigenvalues	Accounts for the relative importance of the global and local configuration of nodes

702

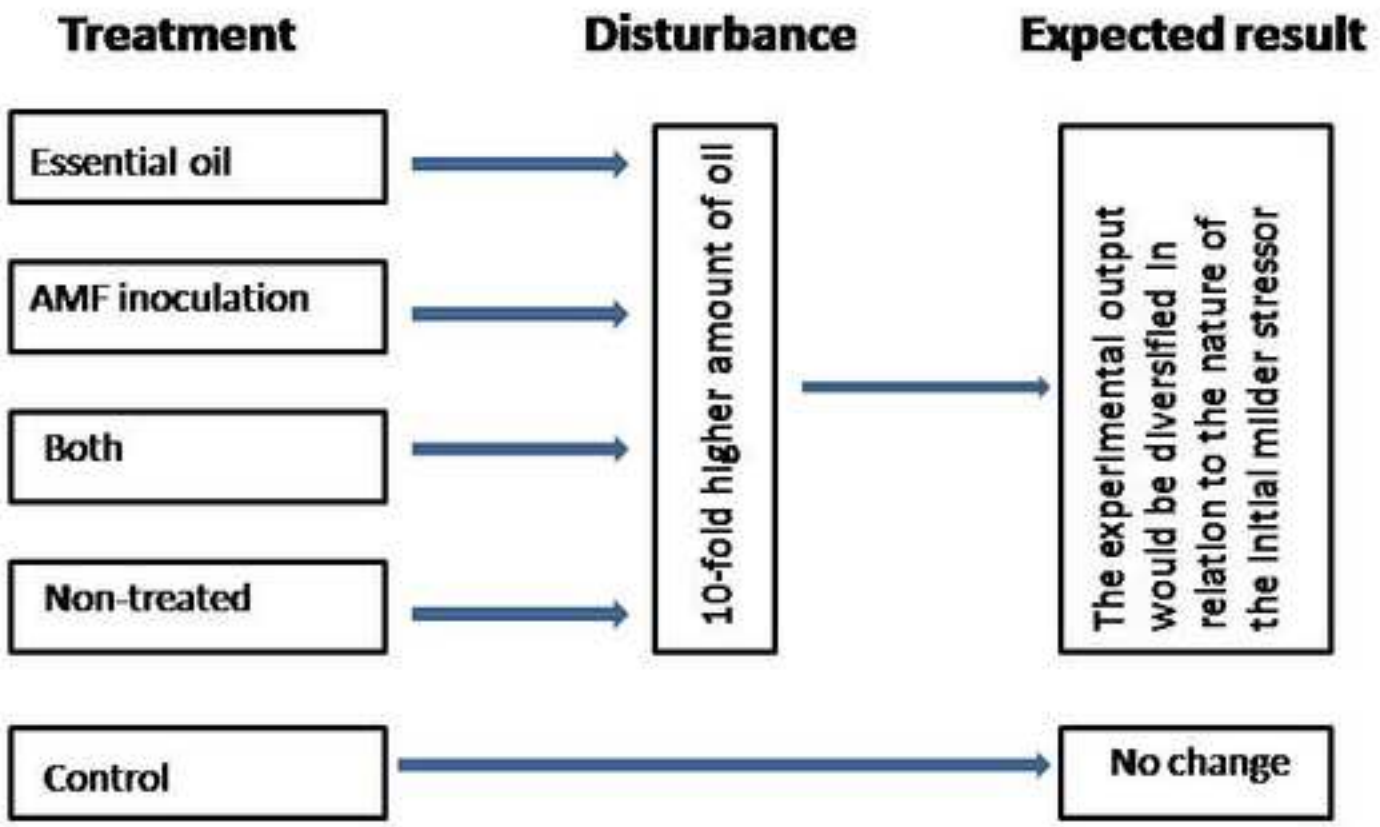
703

704 Table 2. Values of metrics illustrating the architecture of the networks. In the third column
705 average values of the corresponding variables taken from random networks are depicted. The
706 first word in the labels of the first row indicates either AMF inoculation (+) or not (-), the
707 second word indicates either pretreatment with oil (+) or not (-) and the third one indicates
708 either stress (+) or not (-).

	Indices	Random	-AMF-Oil-Str	-AMF-Oil +Str	-AMF +Oil+Str	+AMF-Oil+Str	+AMF + Oil+Str
Cohesion	% Density	16.7	82.4	73.3	52.9	67.1	19.5
	Avg shortest path L	2.64	1.18	1.22	1.54	1.26	3.32
	Compactness	0.39	0.91	0.81	0.75	0.79	0.38
	Nulls	0.75	0.18	0.27	0.47	0.33	0.81
Modularity	R-square	0.22	0.79	0.51	0.63	0.56	0.58
	Transitivity	0.22	0.89	0.92	0.74	0.83	0.67
	E-I	0.54	0.50	0.58	0.28	0.46	0.17
	Clustering coefficient C	0.18	0.91	0.92	0.80	0.86	0.66
	S^{Δ}		11.31	11.01	7.62	10.01	2.92
Centrality	% eigenvector centralization	33.22	7.08	9.02	23.11	12.32	52.99
	$1^{st}/2^{nd}$eigenvalue	1.83	3.59	4.47	1.82	2.94	1.23

709

Figure



Figure

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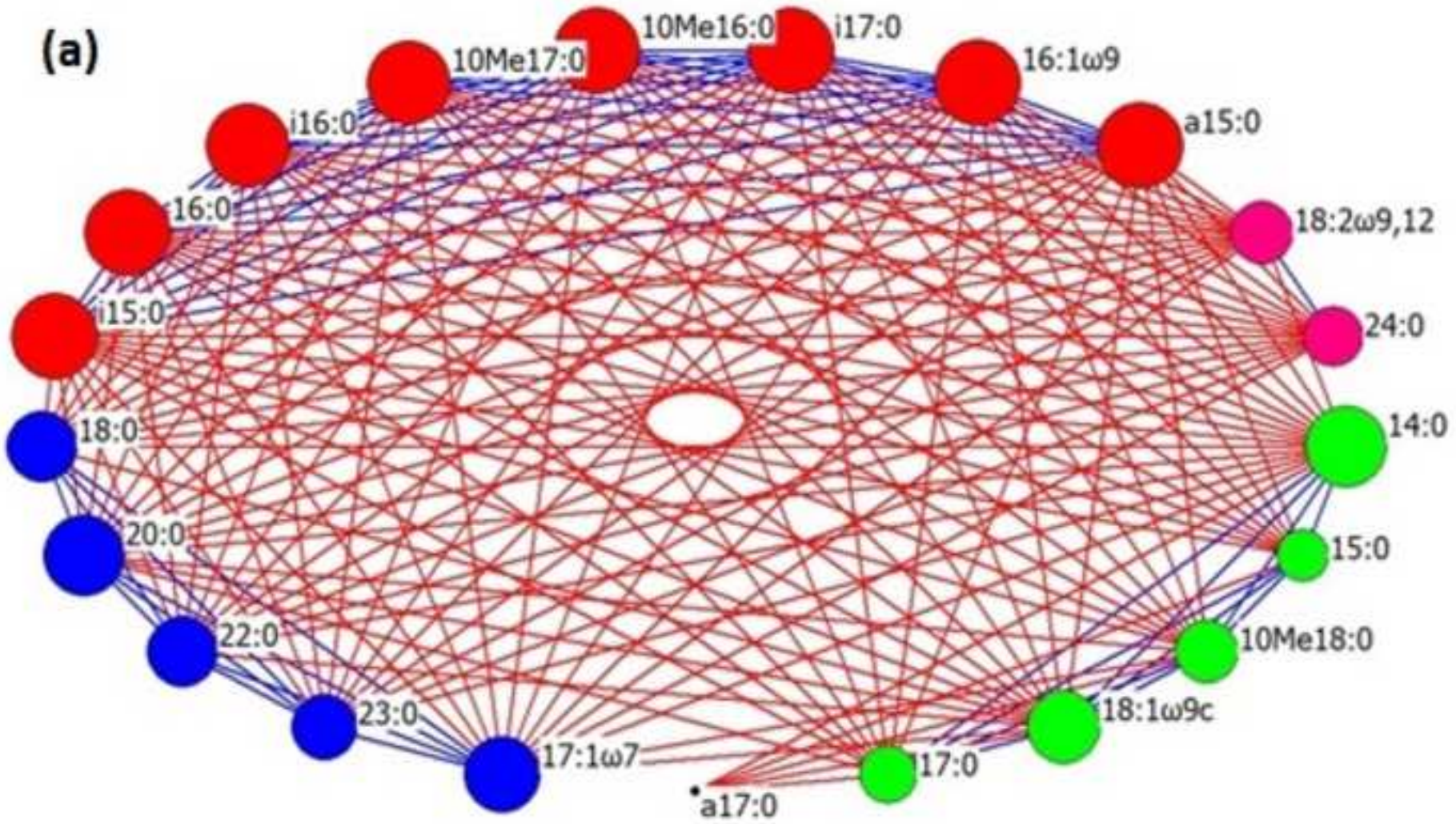

Figure

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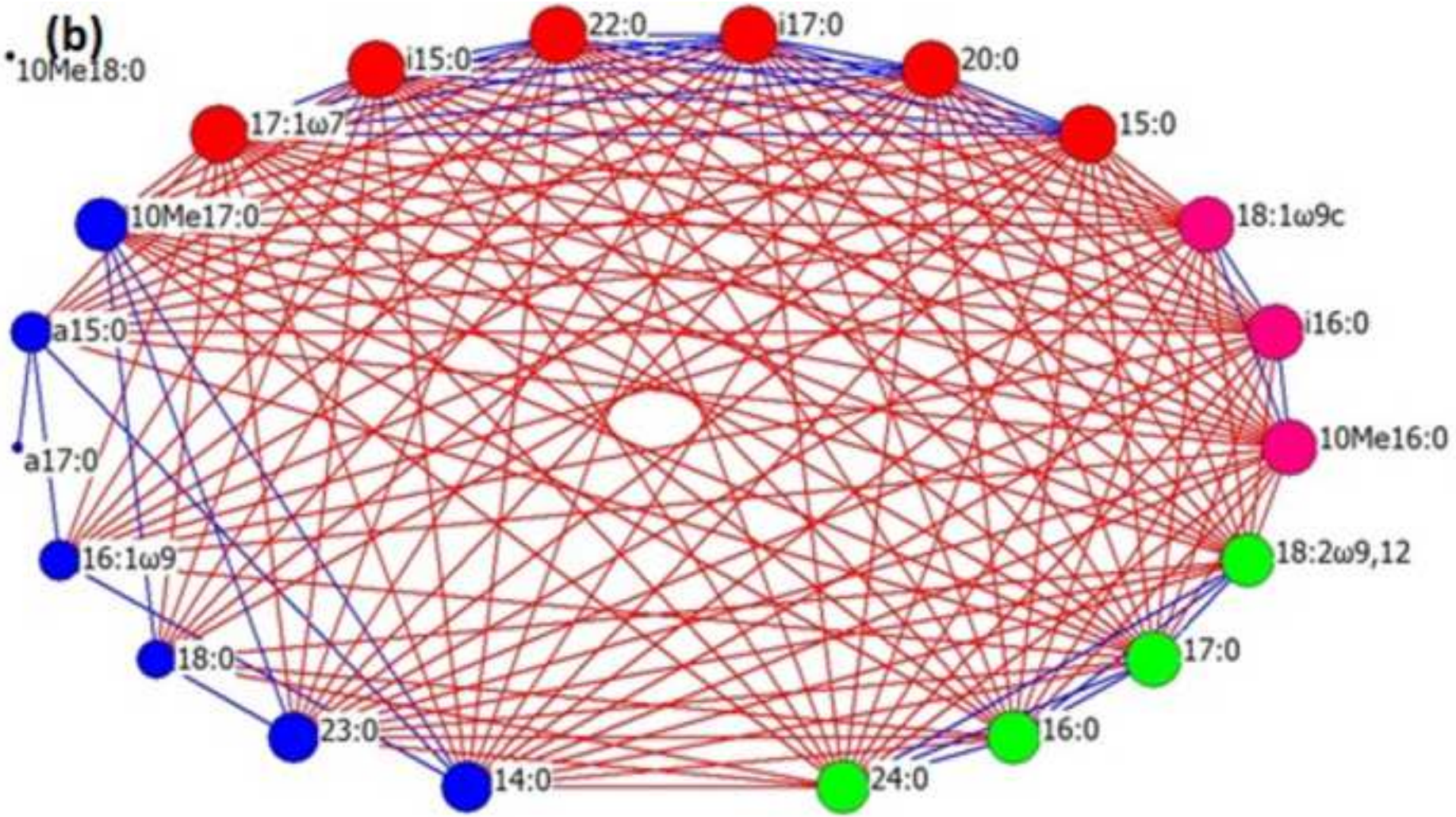

Figure

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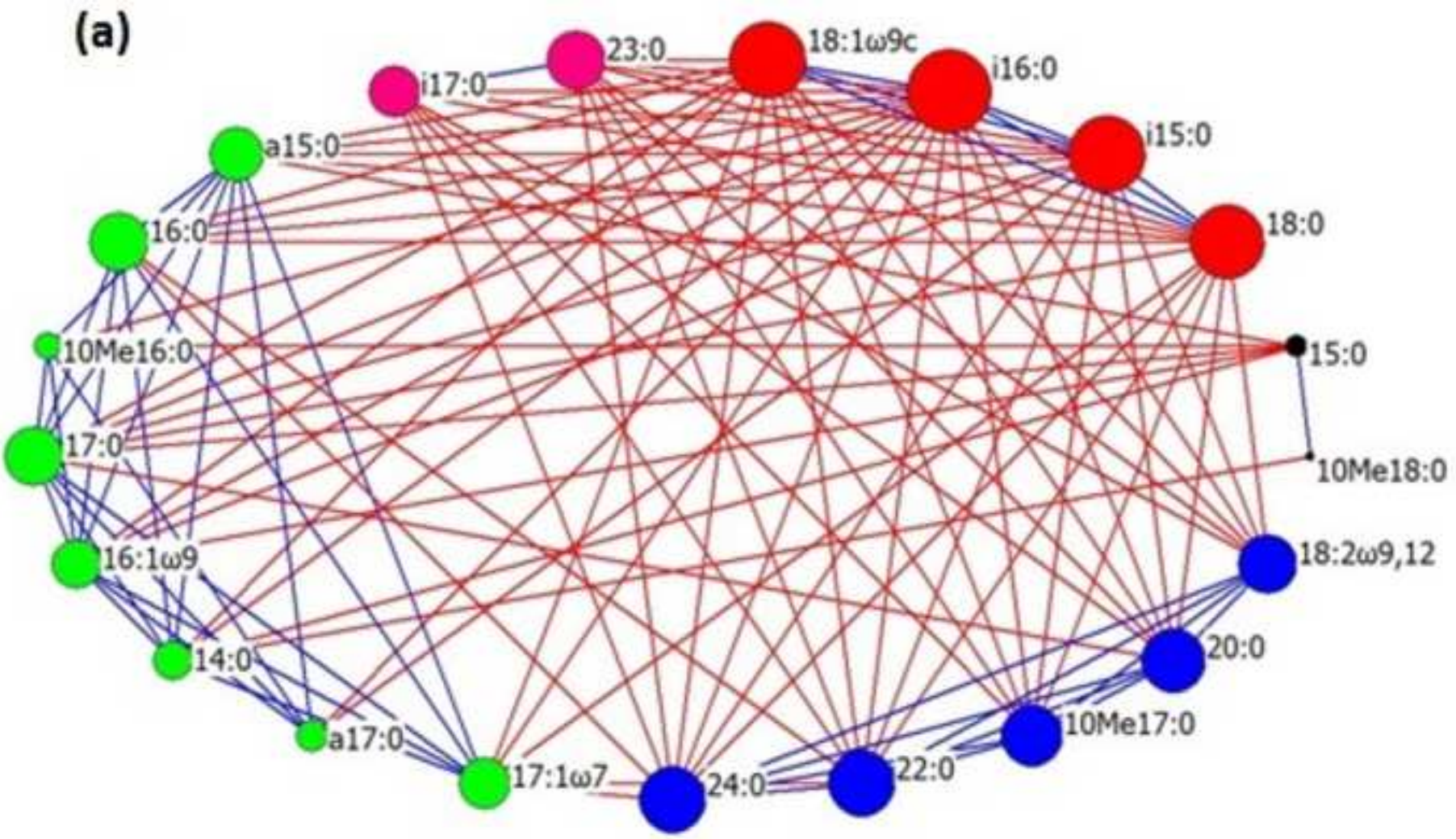

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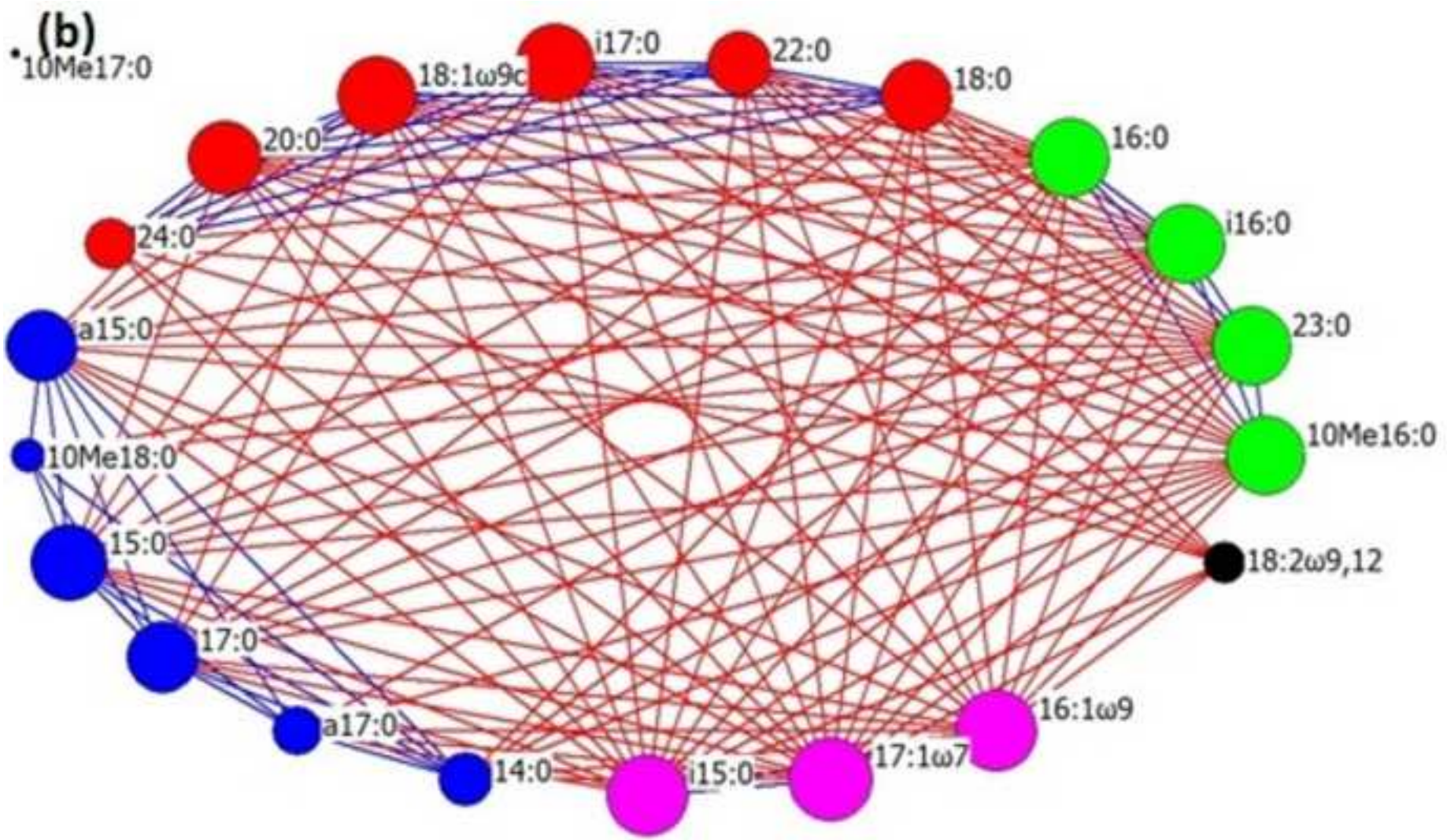

Figure

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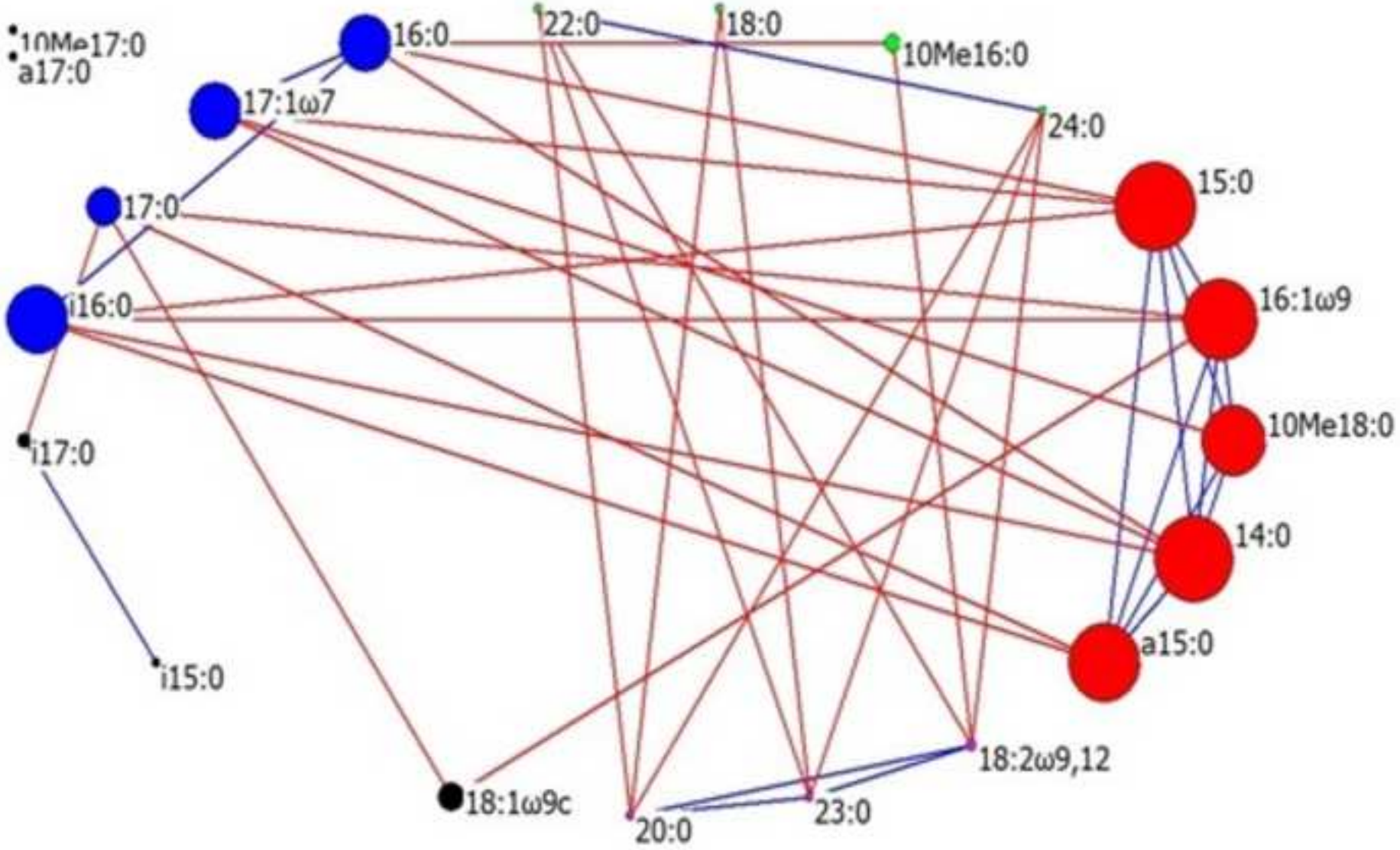

Figure

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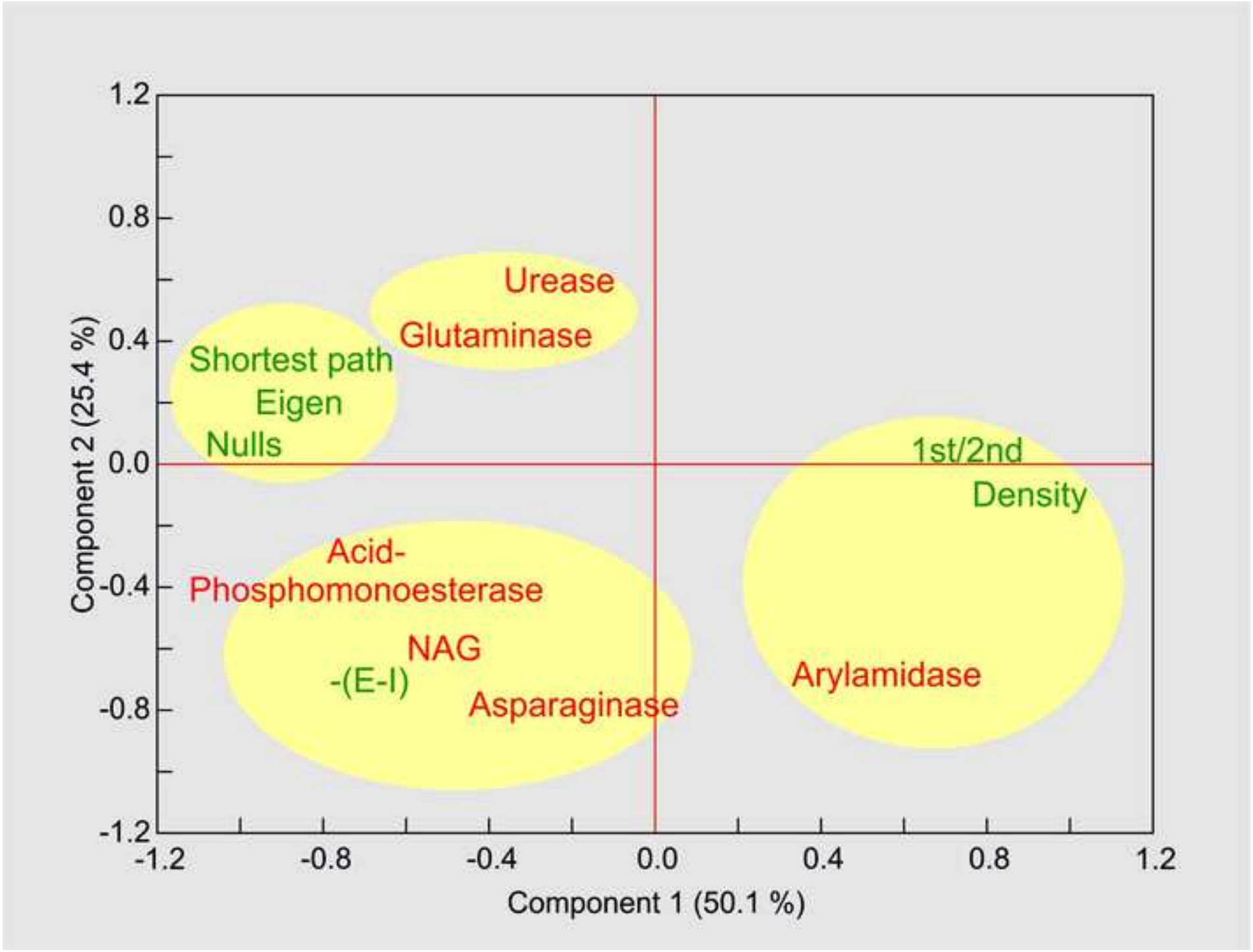

Reply to Reviewers' comments:

Reviewer #1: Line 32 should be non-pretreated

"Done"

Line 40 I don't understand how the results show a "sudden loss of balance" in a system resistant to perturbation.

We replaced the "sudden loss of balance" with "prone to sudden transition towards instability". This type of network characterization follows the suggestion of Sinha (2005).

Line 376 - 378 are directly plagiarized from comments provided by reviewer #1
Lines 376-378. It would be good to apply this statement to the current findings in the following sections. How did oil on oil response differ or not with metal on metal, etc.?

We changed the text presented in lines 376-378 in order to respond to the review's comments (plagiarism and application of statement to our data). Please see the new text in lines 376-401.