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

Stamou, G. P.; . Monokrousos, N; Gwynn-Jones, D.; Whitworth, David; Paptheodorou, E. M.

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

4

5 **G.P. Stamou<sup>1</sup>, N. Monokrousos<sup>2</sup>, D. Gwynn-Jones<sup>3</sup>, D.E. Whitworth<sup>3</sup>, E.M.**  
6 **Papatheodorou<sup>1,4</sup>**

7

8 *<sup>1</sup>International Hellenic University, 14<sup>o</sup> km Thessaloniki-N. Moudania, 57001 Themi,*  
9 *Thessaloniki, Greece*

10 *<sup>2</sup>Department of Soil Science of Athens, Institute of Soil and Water Resources, Hellenic*  
11 *Agricultural Organization-DEMETER, 14123 Athens, Greece*

12 *<sup>3</sup>Institute of Biological Environmental and Rural Sciences, Aberystwyth University,*  
13 *Ceredigion, United Kingdom*

14 *<sup>4</sup>Department of Ecology, School of Biology, Aristotle University of Thessaloniki, 54124*  
15 *Thessaloniki, Greece*

16

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

18 e-mail: [papatheo@bio.auth.gr](mailto:papatheo@bio.auth.gr)

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22 **Abstract**

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

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

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50 **1. Introduction**

51 The global ecological system is described as a complex, self-organized, adaptive and dynamic  
52 structure being represented as a network of interactions that occur between connected nodes  
53 [1, 2]. Local networks within the ecological system can represent any biological hierarchy,  
54 such as species composition or functional groupings [3, 4]. Soil habitats may be viewed as  
55 complex subsystems within the larger ecological system [5], characterized by enhanced  
56 heterogeneity [6] and consisting of superimposed spheres such as the chemical background,  
57 the microbial community, or the enzymatic activity in soil [4].

58 According to Simard [5] the complexity of the soil microbial network is dynamic due  
59 to microbial adaptability because of rapidly evolving microbiota, capable of self-organization  
60 due to the existence of feedback operating across hierarchical spheres. Since soil microbial  
61 communities are in a non-equilibrium state, their composition, activity and abundance can  
62 change due to external influences most of which act as disturbance agents causing  
63 pronounced change in activity and/or abundance of biota. Existing evidence suggests that the  
64 soil microbial community responds differently to various types of disturbances because  
65 microbiota behavior varies in terms of species mortality and the development of the various  
66 microbial species [7]. In this paper we considered that disturbance caused a stress effect  
67 resulting in episodic physiological perturbation [8]. Tobor-Kaplon et al. [9] suggested that  
68 stress-induced changes in the energy budgets of soil organisms can trigger changes in  
69 ecosystem functioning, while Norris et al. [10] and Azarbad et al. [11], among many others,  
70 showed that a wide range of disturbances can exert a strong selective pressure on soil  
71 microbial assemblages. Disturbances are often episodic in natural systems. Philippot et al.  
72 [12] suggested that the response of a microbial community to stress is often dictated by prior  
73 stress exposure. Indeed an initial response to recurrent low-impact events moves the system to  
74 a new state, which modulates responses to a further more severe event [13]. Rillig et al. [13]  
75 suggest that this is due to differences in species' inherent tolerances to stress, the differential  
76 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

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

131

## 132 **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 lycopersicum*) 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<sub>extr</sub> 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**



189 The activities of six soil enzymes were studied. These were *N*-acetyl-glucosaminidase, acid  
190 phosphomonoesterase, urease, asparaginase, glutaminase and arylamidase. *N*-acetyl-  
191 glucosaminidase (NAG) and acid phosphomonoesterase activities were determined according  
192 to Allison and Jastrow [28], as these were modified in order to be applicable for 96-well  
193 microplates. The activity of the two enzymes is presented in units of  $\mu\text{mol pNP g}^{-1} \text{h}^{-1}$ . Urease  
194 activity was determined according to Sinsabaugh et al. [29]. It was expressed as micromoles  
195 of ammonium released per hour per g of soil ( $\mu\text{mol NH}_4^+ \text{g}^{-1} \text{h}^{-1}$ ). Activities of asparaginase  
196 and glutaminase were determined according to Tabatabai [30] with enzyme activity here  
197 expressed as  $\text{mg NH}_4^+ \cdot \text{kg}^{-1} \cdot 2\text{h}^{-1}$ . Arylamidase activity was evaluated according to Acosta-  
198 Martínez and Tabatabai [31]. Activity was expressed as  $\text{mg } \beta\text{-naphthylamide} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ . All  
199 activities were expressed per unit of dry soil.

200

### 201 **2.3 Phospholipid fatty acid analysis**

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

208 The total amount of PLFAs represented the total microbial biomass. The fatty acid  
209 nomenclature was according to Papadopoulou et al. [32]. Overall, 21 fatty acid methyl esters  
210 were identified and considered for further analysis, including the internal standard 19:0; The *i*-  
211 15:0, *a*15:0, 15:0, *i*16:0, *i*17:0 fatty acids were indicators of Gram-positive ( $\text{Gr}^+$ ) bacteria  
212 [33,34,35], the 16:1 $\omega$ 9c was a Gram-negative ( $\text{Gr}^-$ ) bacteria indicator [35], the 16:0, 17:0  
213 were bacteria indicators in general [36] while the 10Me16:0, 10Me17:0, 10Me18:0 fatty acids  
214 were indicators of actinomycetes [37,38]. The sum of these indicators was used to calculate  
215 the bacterial biomass. The 18:1 $\omega$ 9c and 18:2 $\omega$ 9,12 fatty acids were indicators of fungal  
216 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

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

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

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

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

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

273

### 274 **3. Results**

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

288 In the controls (-AMF-Oil-Str), estimated transitivity values were higher than 0.4,  
289 while the average length of the shortest path approximated the Log of the number of nodes  
290 ( $\text{Log}_{21}=1.322$ ) and  $S^A > 1$ . This suggested modularity and small world properties, a trend that  
291 holds also after the stress (-AMF-Oil+Str). In both networks the nodes were partitioned into  
292 four classes of structural equivalence (R-square equals 0.788 and 0.513 respectively,  
293  $P < 0.001$ ). At the whole network scale, positive values of the index E-I were estimated by  
294 taking into account the separation of nodes into clusters of structural equivalence. This  
295 highlighted the superiority of ties between (red coloured lines) versus ties within sub-clusters  
296 (blue coloured lines). Three main classes of structural equivalence were apparent in the -  
297 AMF-Oil-Str network. The first (red circles, density 0.98) contained only markers indicative  
298 of bacteria (five  $\text{Gr}^+$ , two actinomycetes and one  $\text{Gr}^-$ ). Eight members of the first class occupy  
299 the most centralized positions in the network. The second class (blue circles, density 0.92)

300 included four eukaryote and one Gr<sup>-</sup> markers, while the third class (green circles, density  
301 0.95) was not as homogeneous as the first and the second one and included two Gr<sup>+</sup>, one  
302 actinomycetes and two fungal markers.

303         Again, in the network -AMF-Oil+Str three main classes of structural equivalence  
304 were identified, but their composition was less homogeneous than in the previous network.  
305 The first class (red circles, density 0.99) included four markers for bacteria (three Gr<sup>+</sup> and one  
306 Gr<sup>-</sup>) and 2 markers representing eukaryotes. In the second class (blue circles, density 0.35)  
307 there were markers for every microbial guild whereas the third class (green circles, density  
308 0.96) included two Gr<sup>+</sup>, one fungal and one eukaryote markers. Three Gr<sup>+</sup>, one Gr<sup>-</sup> and three  
309 eukaryote markers occupied the more centralized positions.

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

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

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

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

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

355 0.29) contained only bacterial markers (three Gr<sup>+</sup> and one Gr<sup>-</sup>), 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<sup>st</sup> / 2<sup>nd</sup> 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

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

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



407 between 0.915 and 0.742 (all higher than the critical value 0.40; [46]), by the average values  
408 of the shortest path being approximated by the Log of the number of nodes (Log 21=1.322;  
409 [47] and the values of the index  $S^A$ . The modular structuring of networks may suggest  
410 robustness in a context of environmental constraints and deprived resources [47, 51].  
411 Modularity fitted closely at the level of structural equivalence. Consequently, in each  
412 network, the members of each class of structural equivalence would be ecologically  
413 homologous with respect to the exploitation of resources [52] and the response to  
414 environmental constraints [22, 53-55]. This was due to the fact that PLFAs provide strain  
415 profiles rather than being indicative of individual strains. Because of the coarse-scale analysis  
416 of this study, strict taxonomic or functional correspondence with the structurally equivalent  
417 groups was not possible. However, it was possible to loosely associate classes of structural  
418 equivalence with certain guilds. There was a tendency for eukaryotic markers to exhibit  
419 higher values of eigenvalues indicating enhanced centrality in stressed but non-pretreated pots  
420 (-AMF-Oil+Str), while in inoculated and stressed (+AMF-Oil+Str), as well as in control pots  
421 (-AMF-Oil-Str) more central positions tended to be occupied by markers indicating bacteria.  
422 Only three out of the seven markers indicate that Gr<sup>+</sup> bacteria were positioned in the same  
423 cluster together with markers for eukaryotes.

424         The E-I values estimated after the partition of nodes to structural equivalence classes  
425 were positive for all networks. Positive E-I values indicated a prevalence of ties between  
426 classes and enhanced robustness of the global architecture. However, in soil pretreated with  
427 essential oil the E-I values were lowest, indicating enhanced within-class ties and increased  
428 robustness of local configurations. Results show that under pretreatment of soil with *M.*  
429 *spicata* oil and application of a stress of the same nature, the robustness of the local versus the  
430 global architecture of the microbial network is improved. The inter-network differences in  
431 modularity and the contribution of local configurations of nodes and ties to the global  
432 architecture of the networks were likely due to fluctuations in strains' evenness. This is  
433 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<sup>st</sup> / 2<sup>nd</sup> 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 16:1 $\omega$ 9, 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

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

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

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

483

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487

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

489

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664 Anticipating Critical Transitions. *Science* 338, 344-348.  
665 <https://doi:10.1126/science.1225244>
- 666

667 **Figures' Legends**

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

669

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

678

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

687

688 **Figure 4.** Projection of the network of correlations (+AMF+Oil+Str) among microbial PLFAs  
689 on a circle-type plot. The colour of nodes corresponds to different classes of structural  
690 equivalence. The size of nodes accounts for their eigenvector centrality, specifically, the  
691 larger the node, the higher the eigenvector centrality, and the higher the influence of the  
692 corresponding node. The red coloured ties represent links between classes of structural  
693 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 $S^{\Delta}$	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 <sup>st</sup> /2 <sup>nd</sup> eigenvalues	Accounts for the relative importance of the global and local configuration of nodes

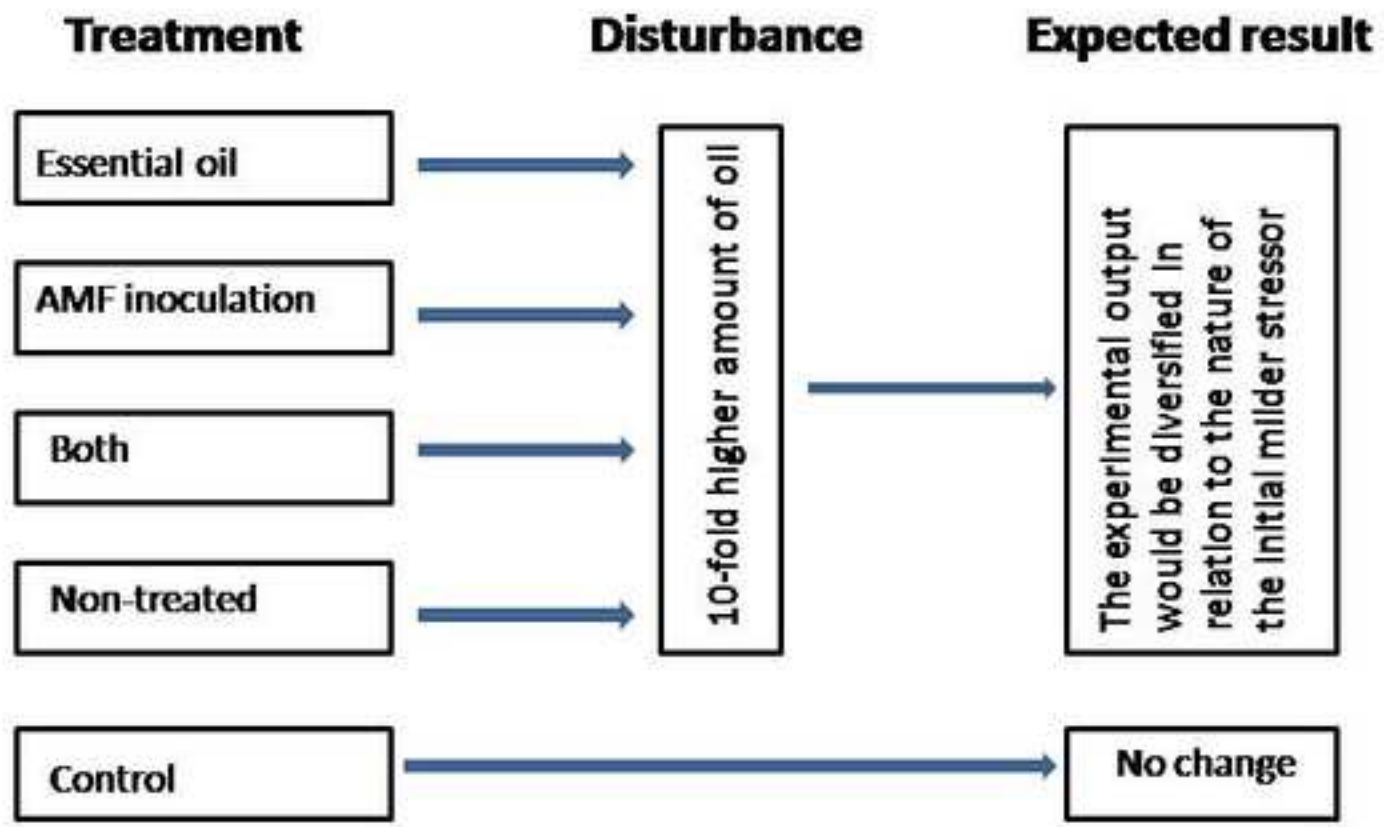
702

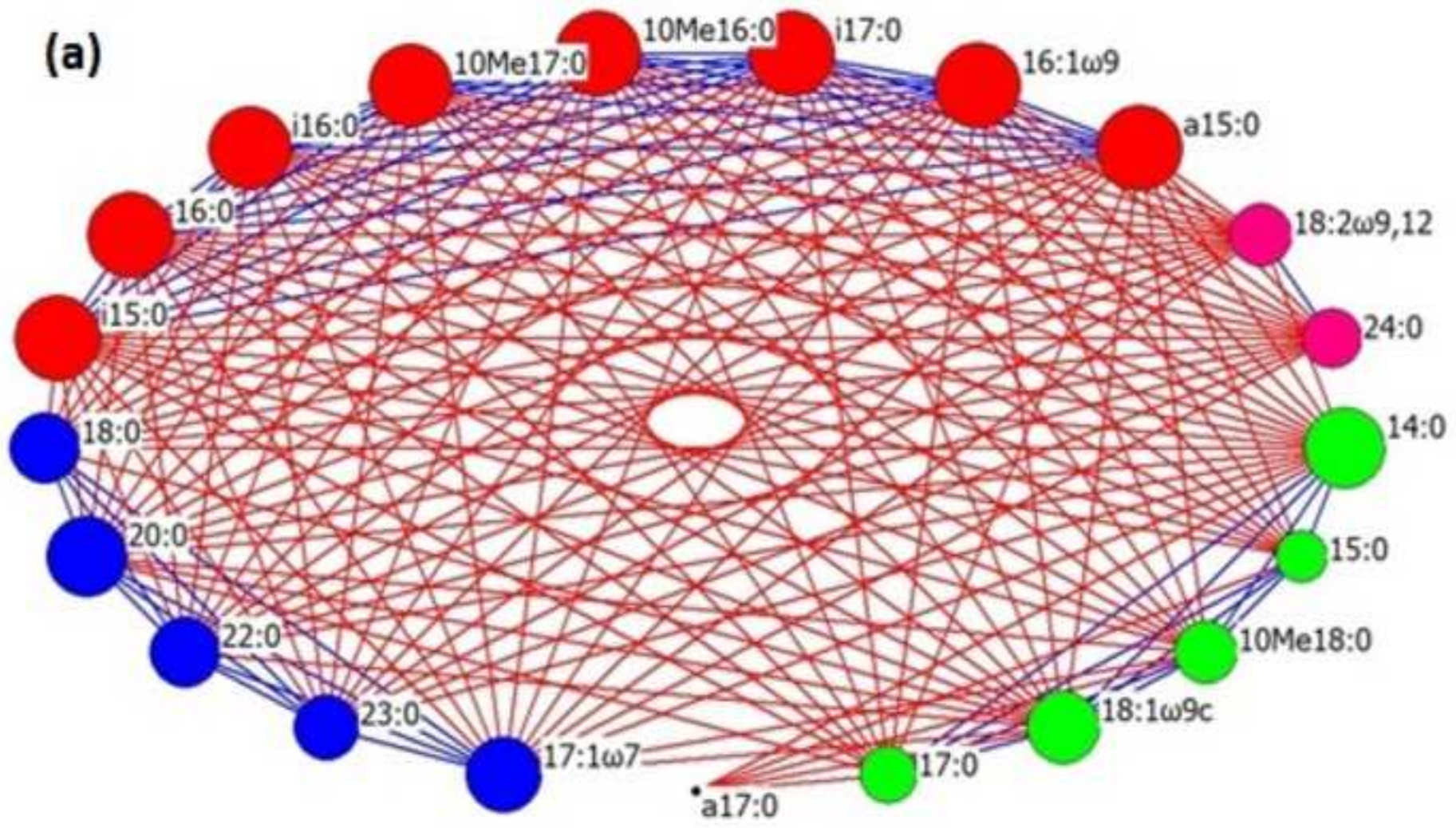
703

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 (-).

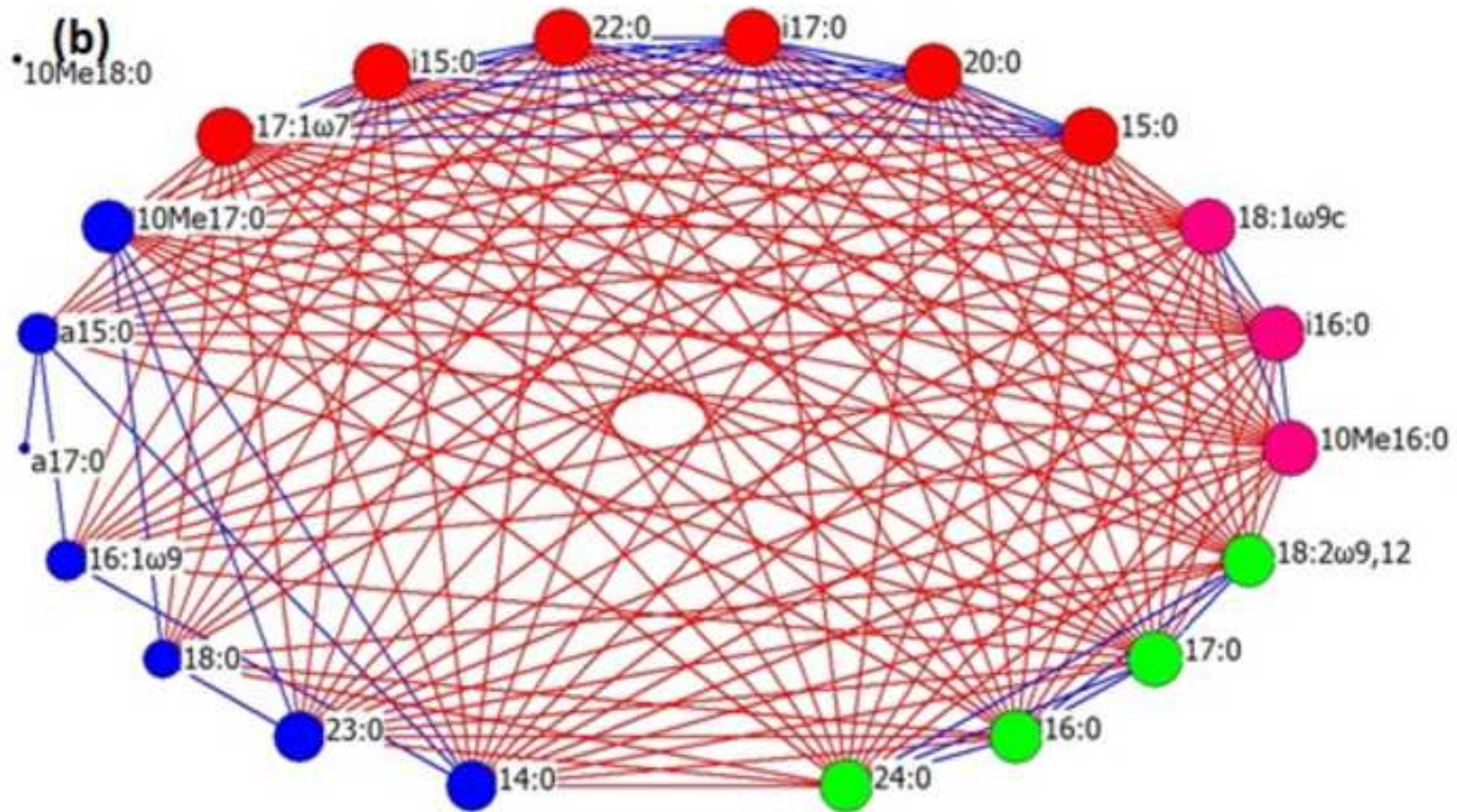
	Indices	Random	-AMF- Oil-Str	-AMF- Oil +Str	-AMF +Oil+Str	+AMF- Oil+Str	+AMF + Oil+Str
Cohesion	% Density	16.7	82.4	73.3	52.9	67.1	19.5
	Avg shortest path L	2.64	1.18	1.22	1.54	1.26	3.32
	Compactness	0.39	0.91	0.81	0.75	0.79	0.38
	Nulls	0.75	0.18	0.27	0.47	0.33	0.81
Modularity	R-square	0.22	0.79	0.51	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 <sup>Δ</sup>		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 <sup>st</sup> /2 <sup>nd</sup> eigenvalue	1.83	3.59	4.47	1.82	2.94	1.23

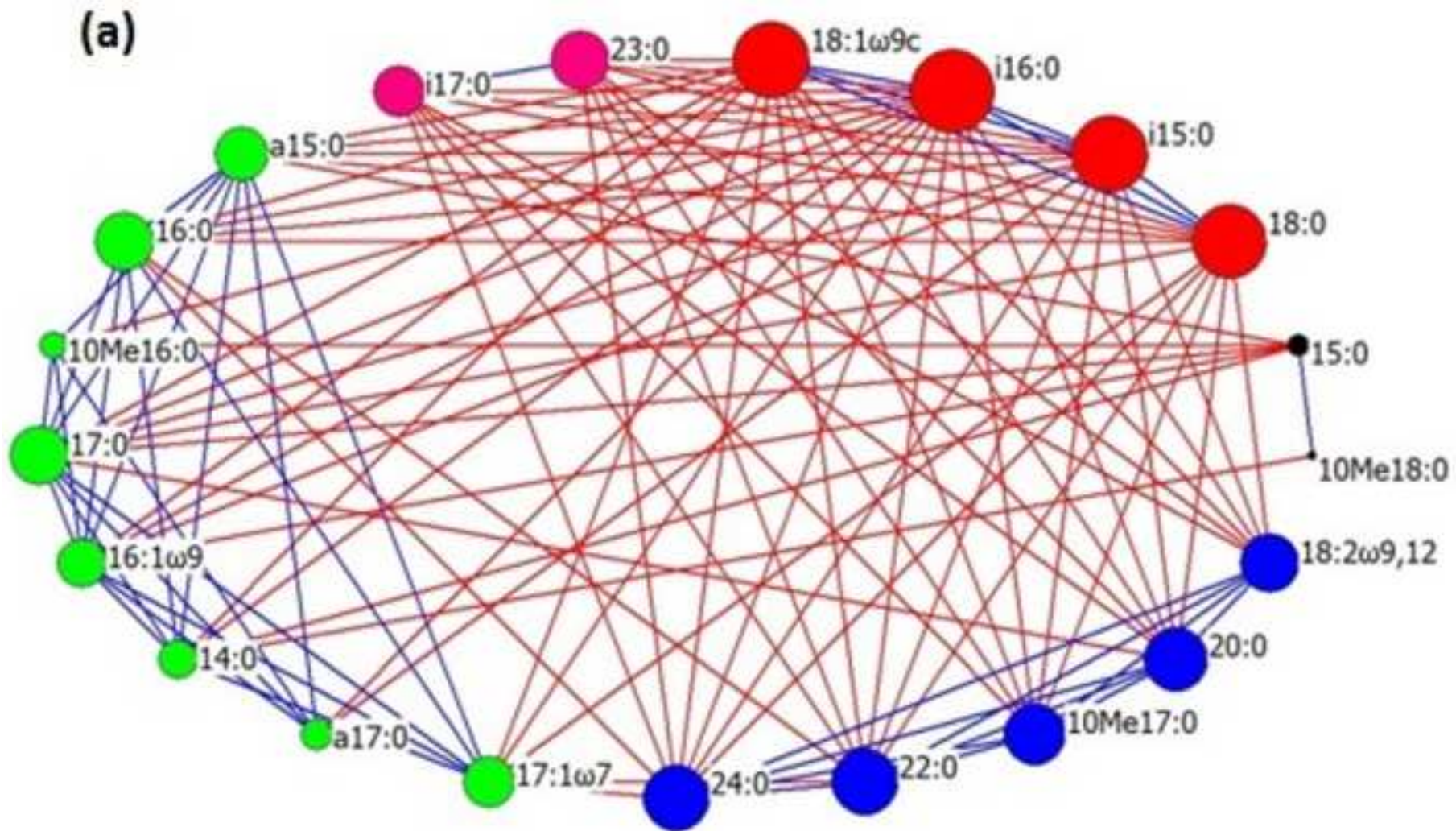
709



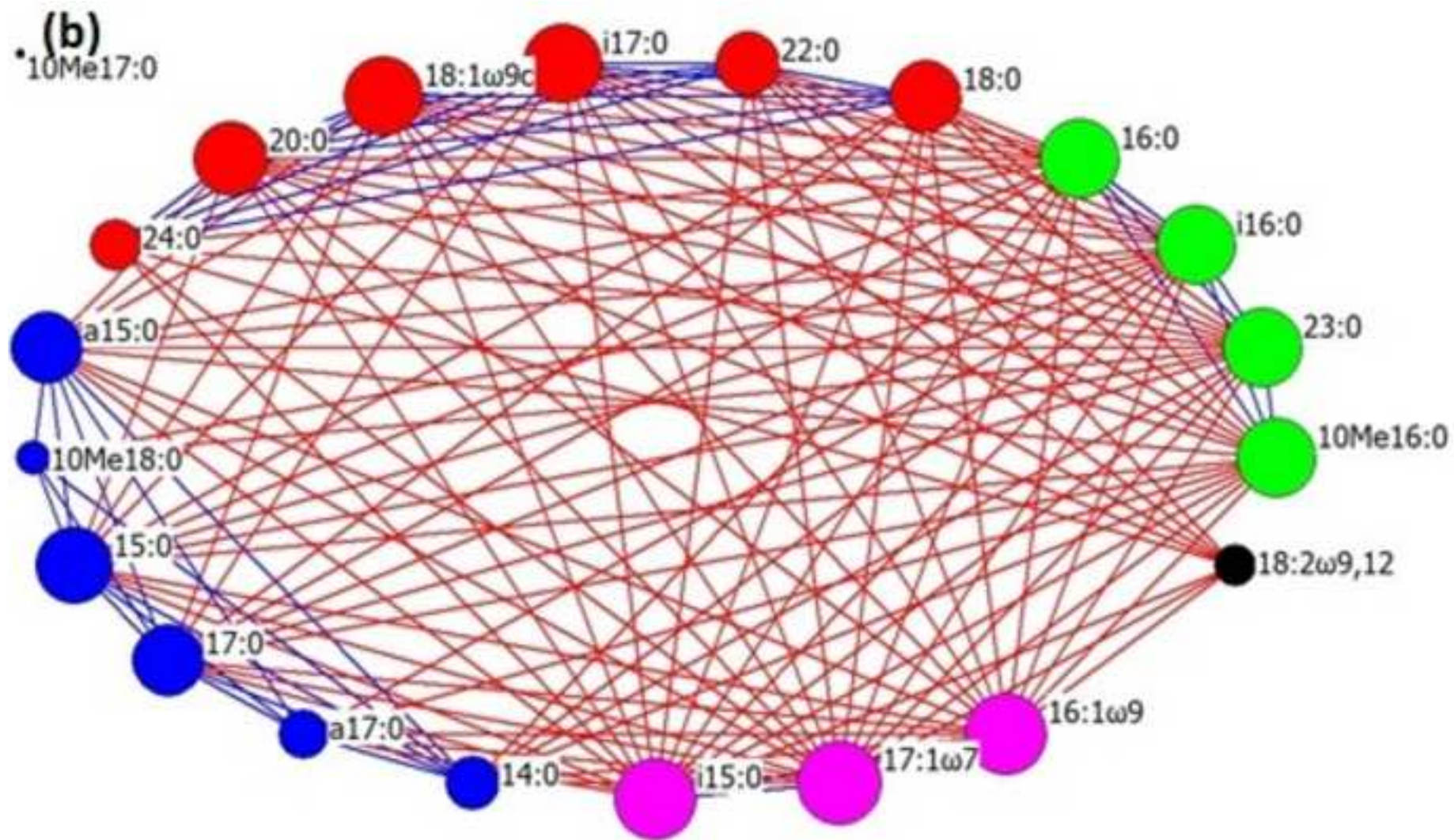


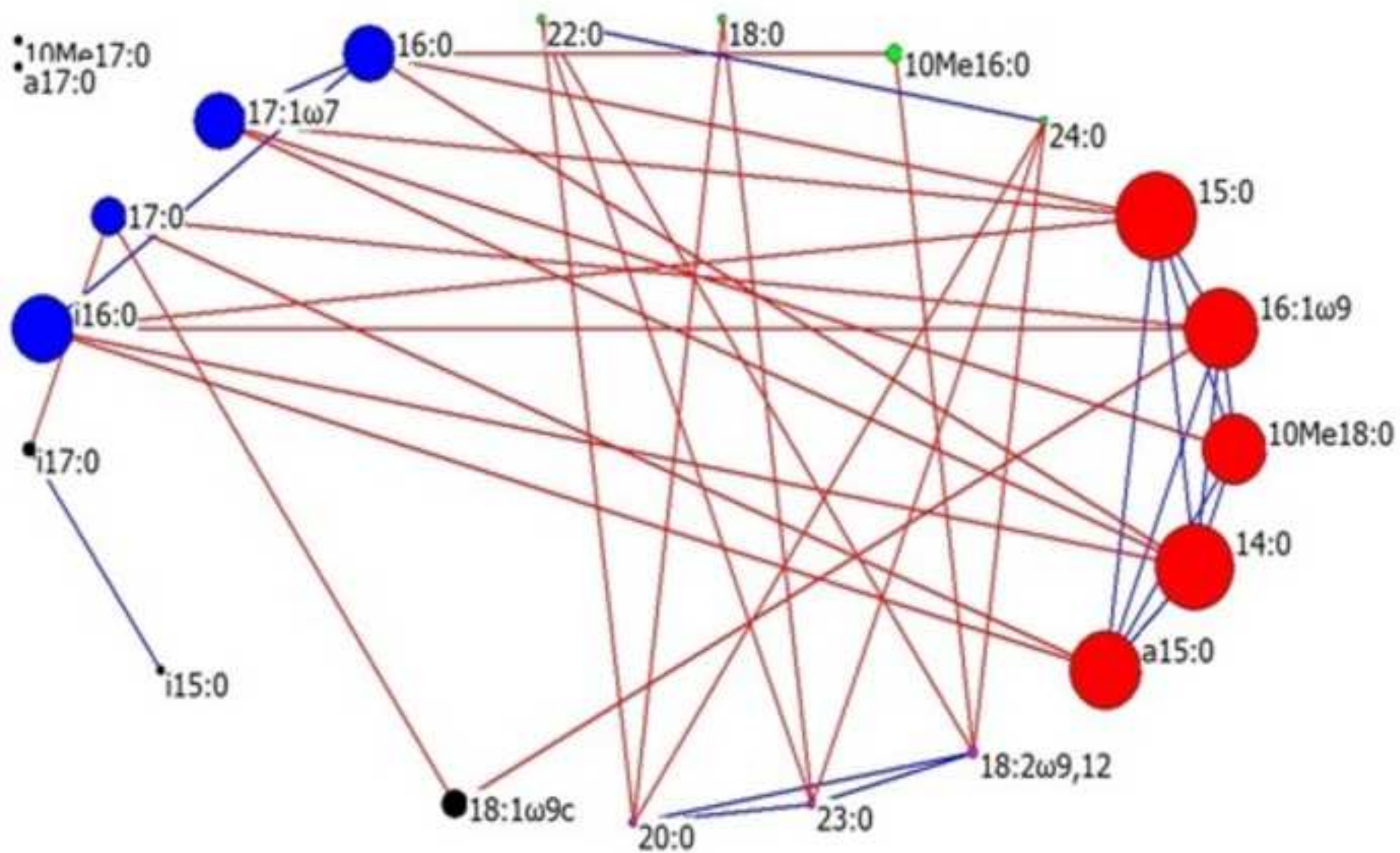


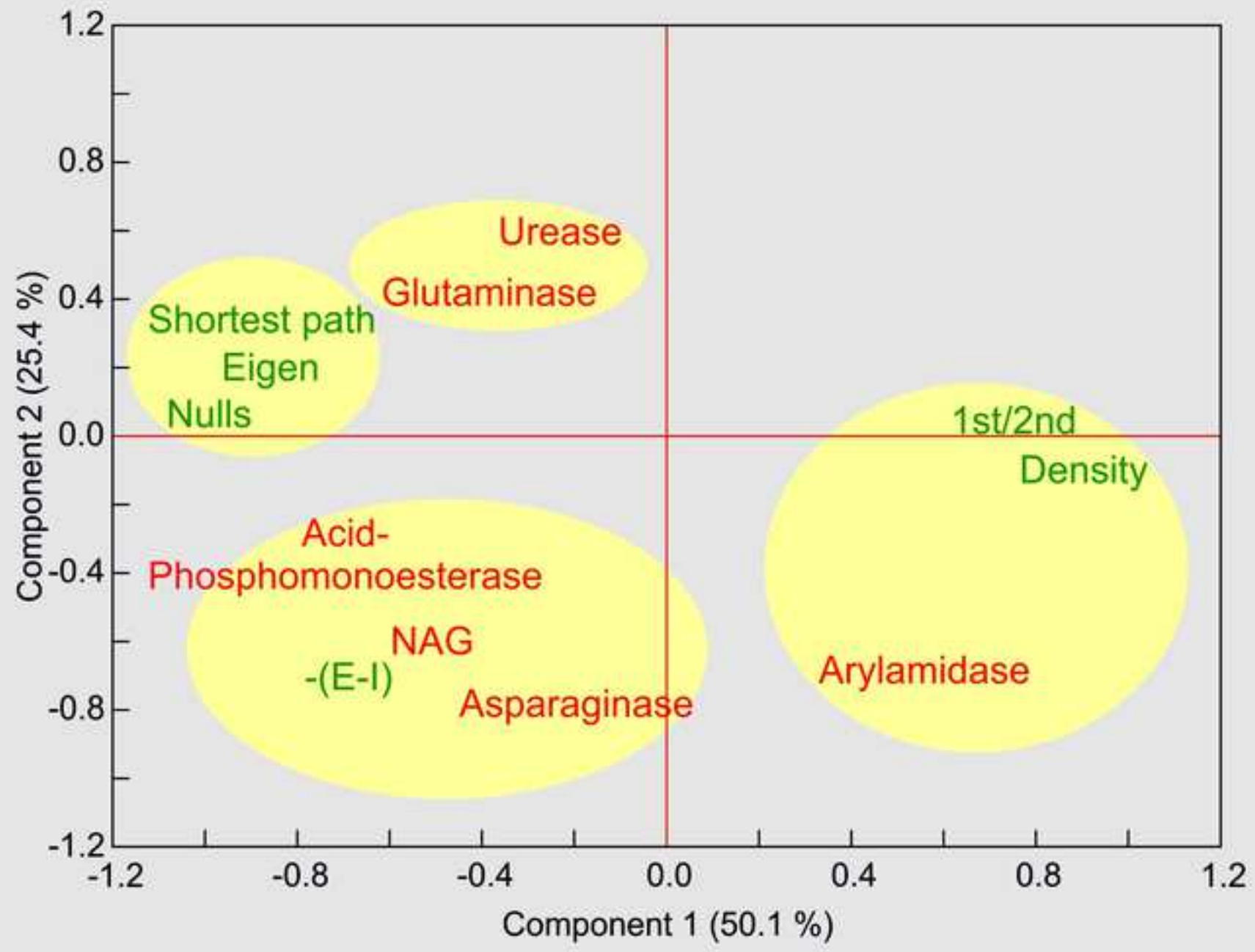














**Reply to Reviewers' comments:**

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.*