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

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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

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**

2.1. Experimental design

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

Half of the AMF inoculated pots (six) along with the six non-inoculated pots were treated with *M. spicata* (spearmint) essential oil. The spearmint oil was supplied by Etherio, Research and Commerce, Eratera, Greece and it was pure essential oil produced after distillation of *M. spicata* plants. The oil was added at a weekly rate of 1.33 ml per pot, for a period of one month. The major compounds of *M. spicata* oil were carvone 63.9% and limonene 13.3% followed by 1,8-cineole, β-pinene, myrcene and α-pinene in percentages 7.1, 2.8, 2.4 and 1.4%, respectively [23]. The experiment was conducted in a glasshouse under natural light conditions for a two-month period (from mid-June to mid-August). During the 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 μmol pNP g^{-1} 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 (μmol NH_4^+ g^{-1} h^{-1}). Activities of asparaginase

196   and glutaminase were determined according to Tabatabai [30] with enzyme activity here

197   expressed as mg NH_4^+ $.kg^{-1}.2h^{-1}$. Arylamidase activity was evaluated according to Acosta-

198   Martínez and Tabatabai [31]. Activity was expressed as mg *β*-naphthylamide. $kg^{-1}.h^{-1}$. 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, a15:0, 15:0, i16:0, i17:0 fatty acids were indicators of Gram-positive (Gr^+) bacteria

212   [33,34,35], the 16:1ω9c was a Gram-negative (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 18:1ω9c and 18:2ω9,12 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^Δ 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^Δ_g) is higher than that for an equivalent random graph ($C^\Delta_g \gg$

258   C^Δ_{rand}), i.e. if $S^\Delta = (C^\Delta_g/C^\Delta_{rand})/(L_g/L_{rand}) > 1$

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 eigencentrality 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

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^Δ>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 1st/ 2nd 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^Δ>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)

300 included four eukaryote and one Gr⁻ 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⁺, 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⁺ and one

306 Gr⁻) 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⁺, one fungal and one eukaryote markers. Three Gr⁺, one Gr⁻ and three

309 eukaryote markers occupied the more centralized posistions.

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 $S^\Delta > 1$.

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 stessed treatment. The difference in the values of the

325 ratio 1ˢᵗ / 2ⁿᵈ 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 dististinguished in the -

330   AMF+Oil+Str network. The first (red circles, density 0.95) included two Gr$^+$, 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$^+$, two Gr$^-$, one actinomycetes)

334   dominated, with one fungal marker. The most centralized positions were occupied by four

335   eukaryotic, three Gr$^+$ 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$^+$ and one fungal marker were also included. In the

339   second class (blue circles) there were four Gr$^+$, along with one actinomycete and one fungal

340   markers. In the third class, there were two Gr$^+$, one actinomycete and one eukaryote markers.

341   Finally, the fourth class comprised one Gr$^+$ and two Gr$^-$ markers. Two Gr$^-$ markers followed

342   by four Gr$^+$ 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 1st / 2nd 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 netwotk. In the

353   first (red circles, density 0.90) there were four bacterial markers (two Gr$^+$, one Gr$^-$ 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^+ 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 1st / 2nd 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^Δ. 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^+ 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 1st / 2nd 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

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 phosphomoesterase 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

487

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

489

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666

Figures' Legends

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^{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

(a)

(b)
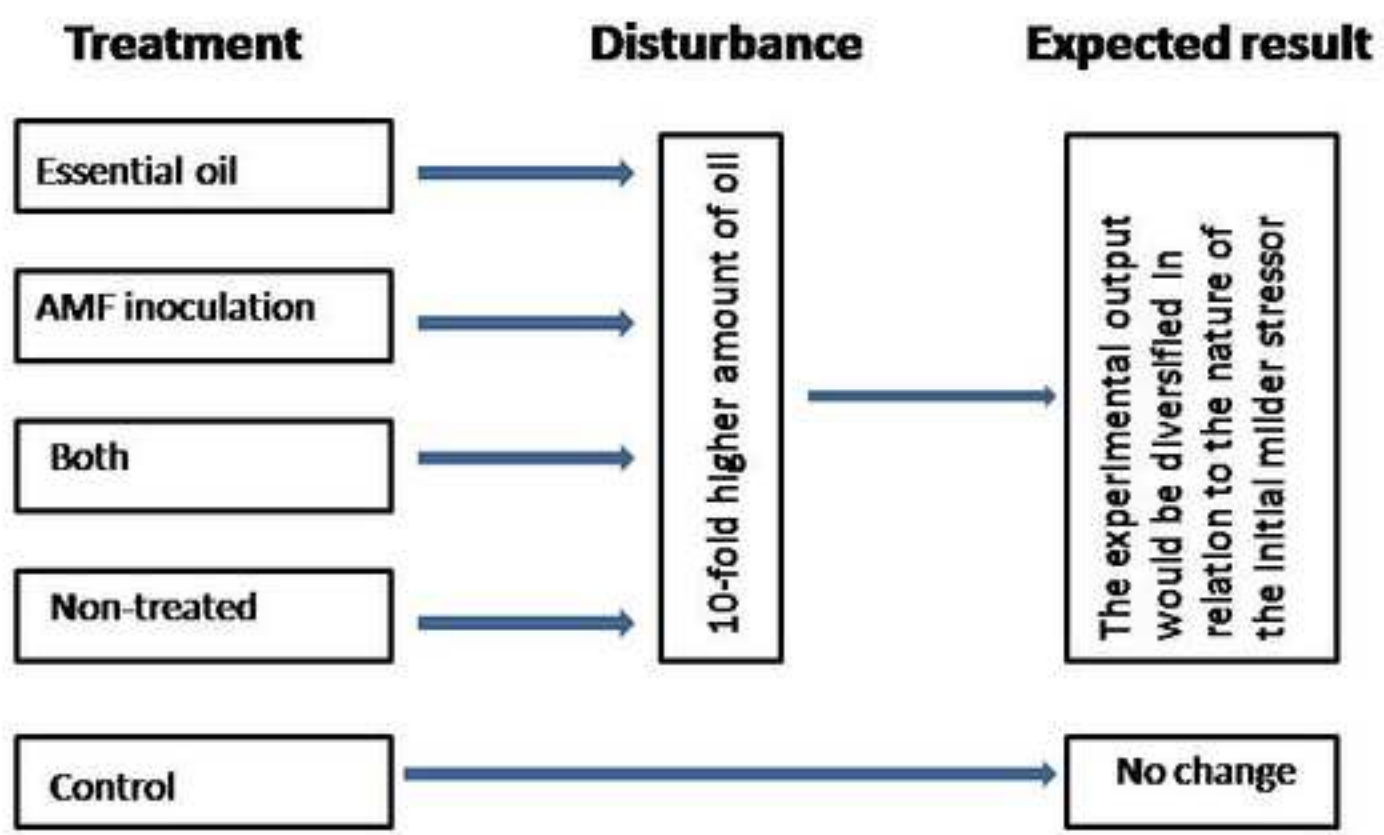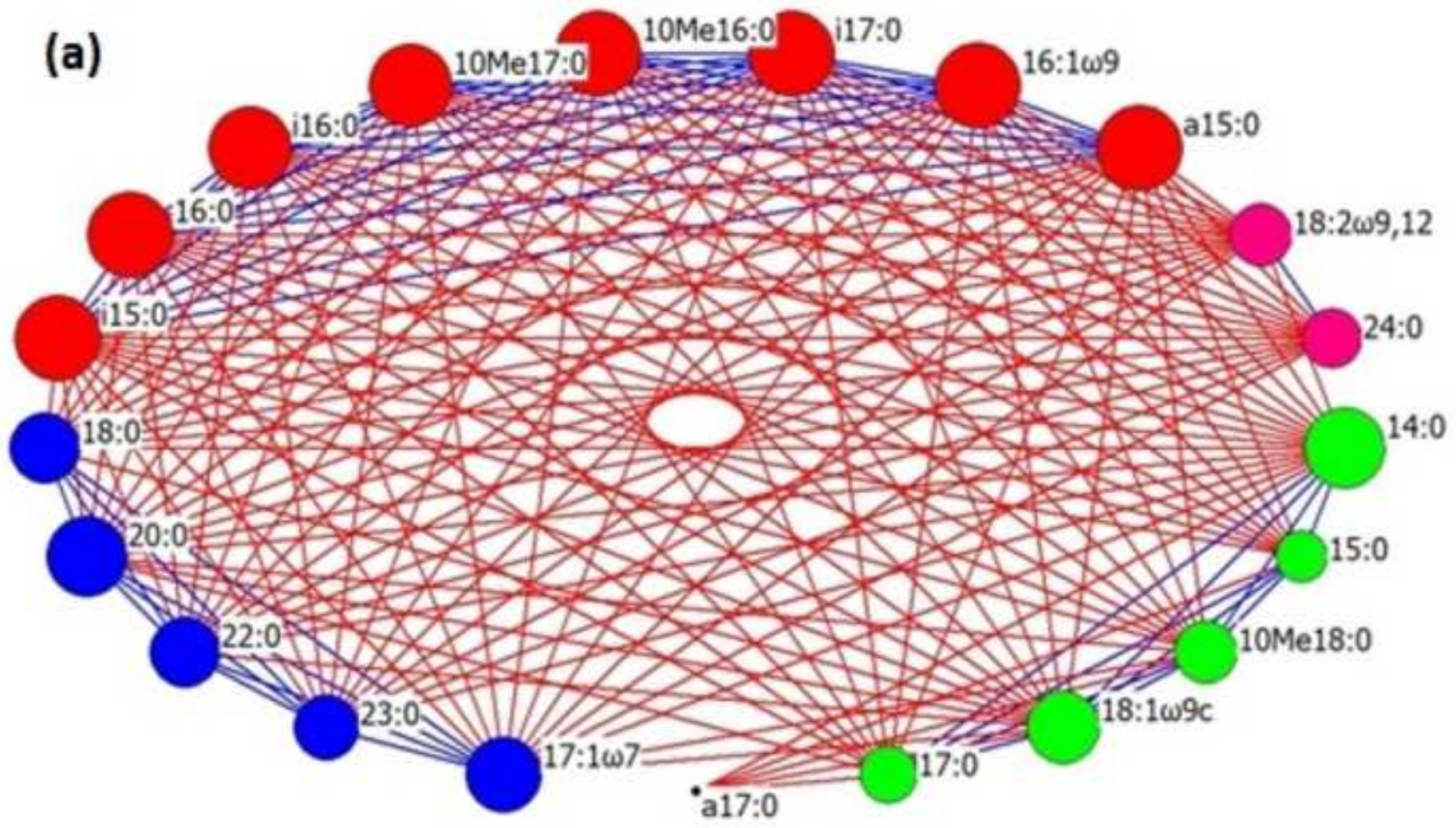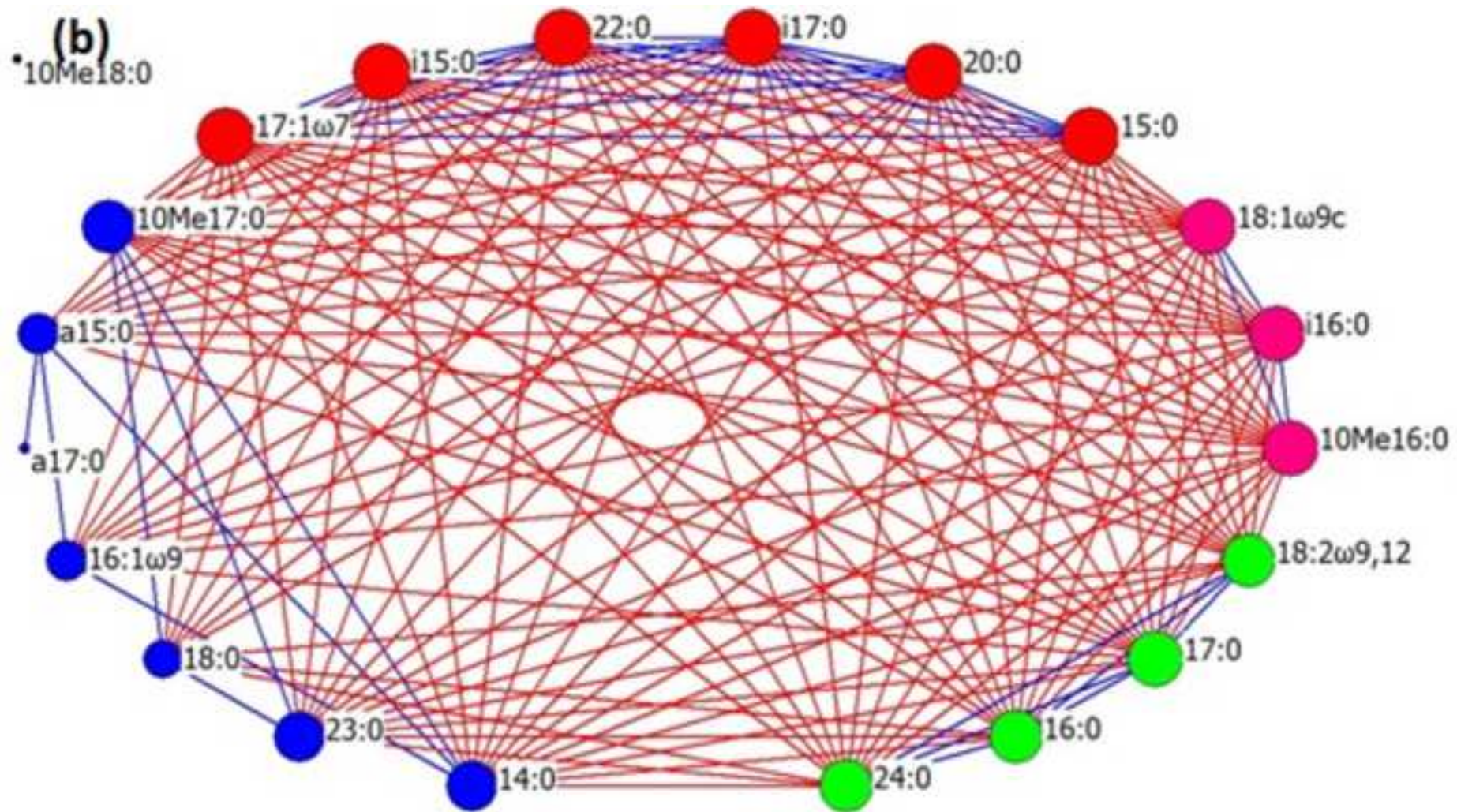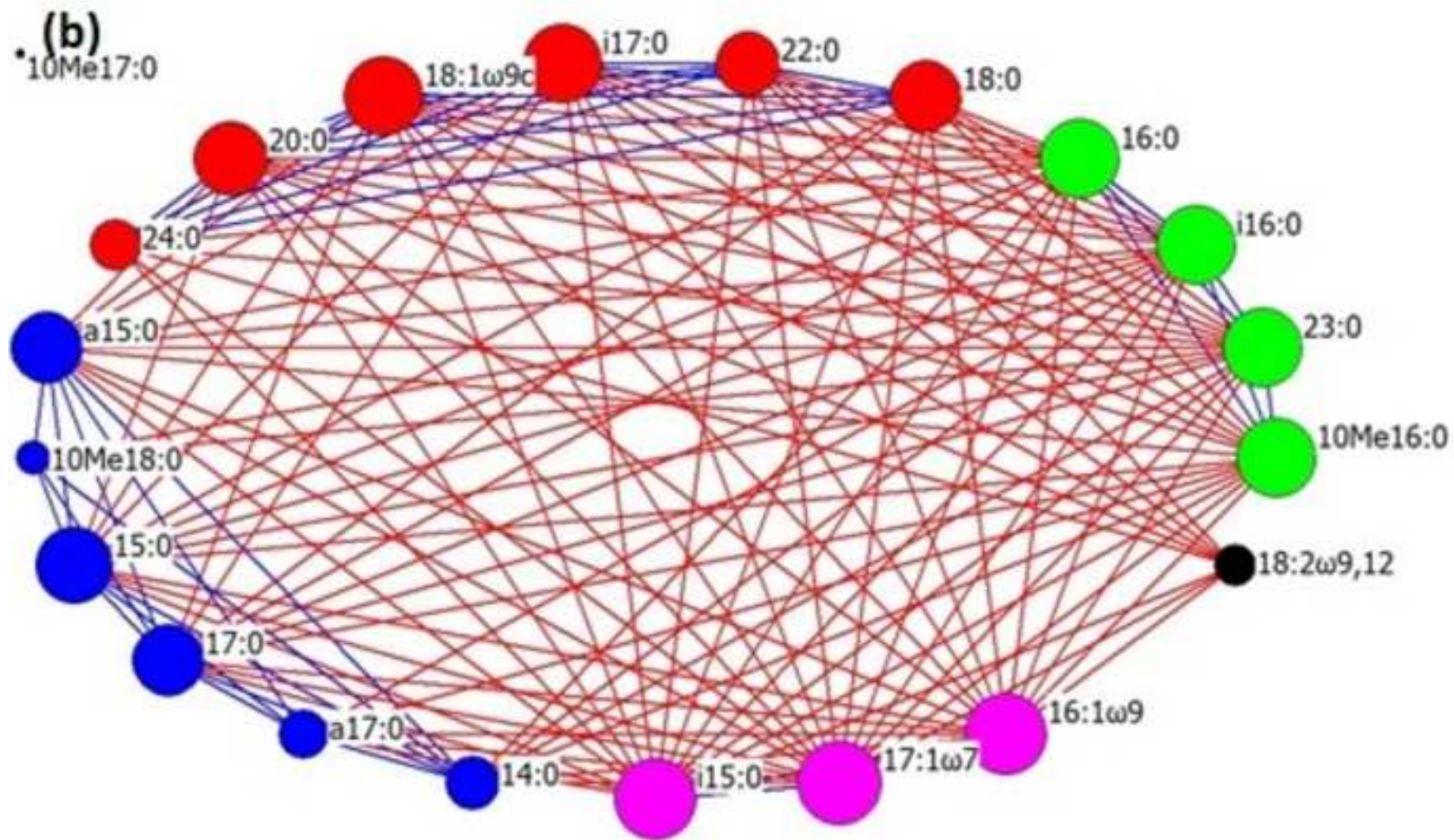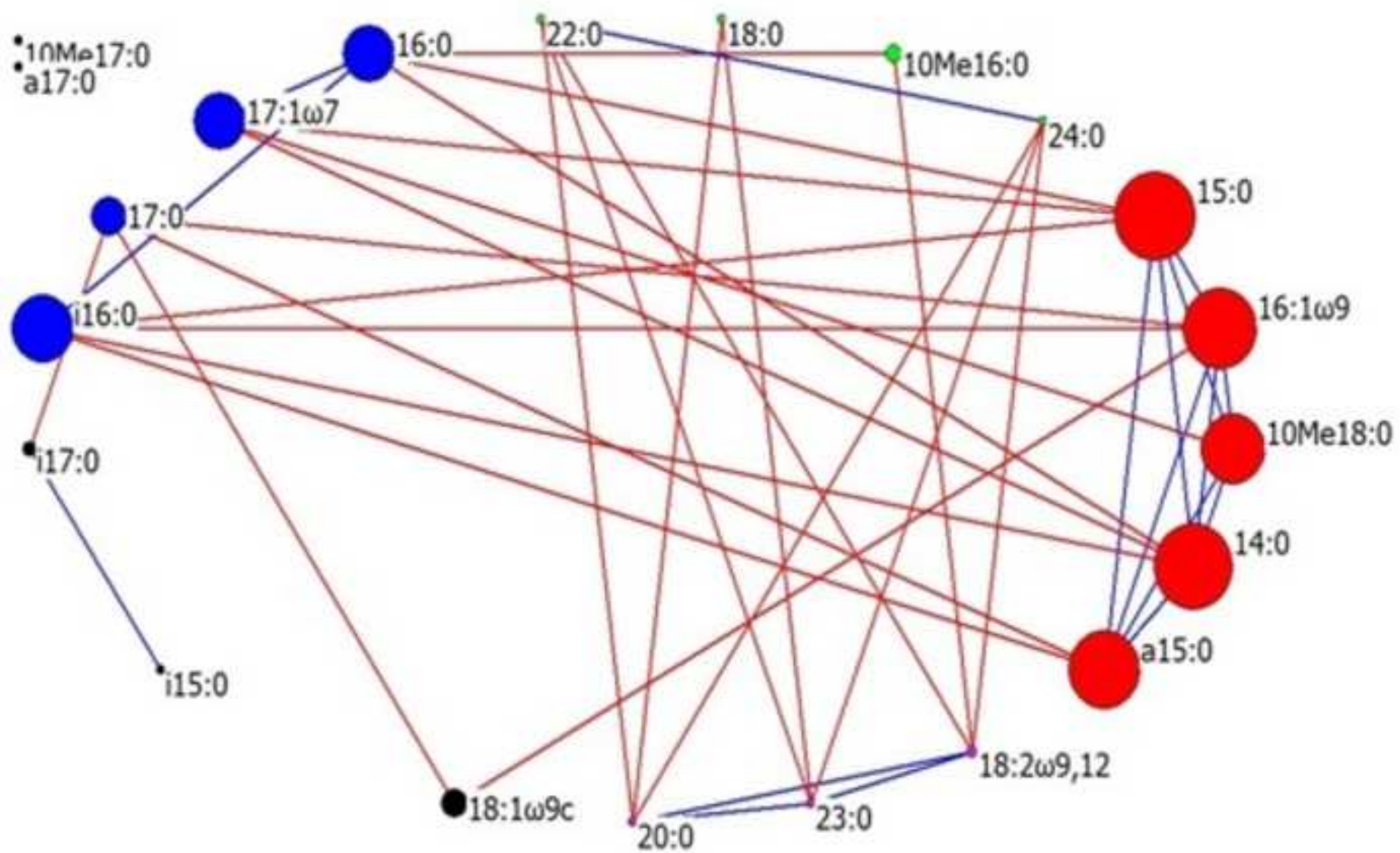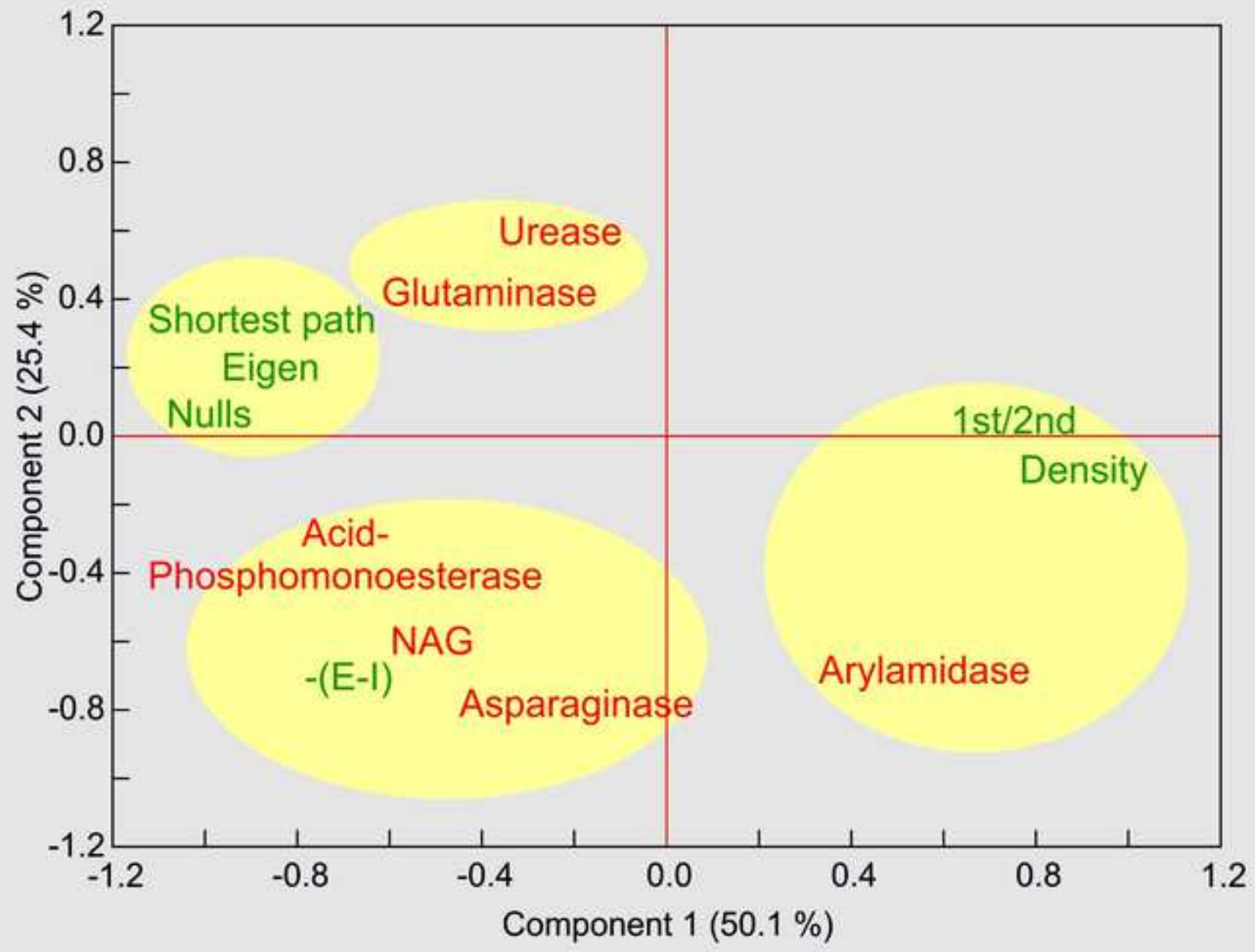
Reply to Reviewers' comments:

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.