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Expanding the Chemical Diversity through Microorganisms Co-culture: Current Status and Outlook

Divya Arora^{1,3,4}, Prasoon Gupta^{2,3}, Sundeep Jaglan^{1,3}, Catherine Roullier⁴, Olivier Grovel⁴,
Samuel Bertrand^{4*}

¹Microbial Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Jammu-180001, India; ²Natural Product Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Jammu-180001, India; ³Academy of Scientific and Innovative Research (AcSIR), Jammu Campus, Jammu – 180001, India; ⁴Groupe Mer, Molécules, Santé-EA 2160, Faculté des Sciences pharmaceutiques et biologiques, Université de Nantes, 9 rue Bias, BP 53508, F-44035 Nantes Cedex 01, France

*Corresponding Author: Samuel Bertrand, PhD

Groupe Mer, Molécules, Santé-EA 2160, Faculté des Sciences pharmaceutiques et biologiques, Université de Nantes, 9 rue Bias, BP 53508, F-44035 Nantes Cedex 01, France; Tel: +33 (0)2 51 12 56 89; Correspondence: samuel.bertrand@univ-nantes.fr (S. Bertrand)

Abstract

Natural products (NPs) are considered as a cornerstone for the generation of bioactive leads in drug discovery programs. However, one of the major limitations of NP drug discovery program is “rediscovery” of known compounds, thereby hindering the rate of drug discovery efficiency. Therefore, in recent years, to overcome these limitations, a great deal of attention has been drawn towards understanding the role of microorganisms’ co-culture in inducing novel chemical entities. Such induction could be related to activation of genes which might be silent or expressed at very low levels (below detection limit) in pure-strain cultures under normal laboratory conditions. In this review, chemical diversity of compounds isolated from microbial co-cultures, is discussed. For this purpose, chemodiversity has been represented as a chemical-structure network based on the “Tanimoto Structural Similarity Index”. This highlights the huge structural diversity induced by microbial co-culture. In addition, the current trends in microbial co-culture research are highlighted. Finally, the current challenges (1 - induction monitoring, 2 - reproducibility, 3 - growth time effect and 4 - up-scaling for isolation purposes) are discussed. The information in this review will support researchers to design microbial co-culture strategies for future research efforts. In addition, guidelines for co-culture induction reporting are also provided to strengthen future reporting in this NP field.

Keywords: Chemical diversity, Induction, Microbial co-culture

1. Introduction

Microorganisms have played a significant role as sources of specialized metabolites with a vast chemical diversity (Nai and Meyer, 2018). Currently, they are taking an even greater place in drug discovery programs due to the ease of sustainable microbial biomass production and resourcing through fermentation. However, as from other sources of natural products (NPs), chemists are currently struggling with the continuous rediscovery of known chemical entities (Gonzalez-Sabin, 2012). This is in contradiction with the high chemical diversity revealed by genome sequencing of microorganisms which revealed the huge difference between the number of NPs gene clusters present in such sequences with respects to the number of observed NPs produced in standard laboratory conditions (the latter being much less) (Pettit, 2011). To overcome such limitation, various approaches have been developed which could be considered as culture and non-culture-based strategies. The latter approaches (non-culture-based) are related to molecular biology and correspond to the specific engineering of organisms able to produce the desired molecules (*i.e.*, heterologous expression (Gomez-Escribano and Bibb, 2014), homologous activation (Zhang, B. et al., 2017), mutagenesis (Li et al., 2016)). Consequently, various culture-based strategies, such as the OSMAC approach (One Strain Many Compounds), epigenetic modification, co-culture, etc. have also been exploited to expand chemical diversity (Bode et al., 2002; Williams et al., 2008). In such cases, the presence of biological and chemical stimuli results in the biosynthesis of diverse NPs by unlocking cryptic pathway expression (Goodwin et al., 2015). Among these approaches, microbial co-culture, consists of growing multiple organisms together in a same confined environment has gained tremendous attention. It allows the microorganisms to initiate chemical interactions and results in the production of NPs that are not observed in pure-strain cultures such as pheromones, defence molecules or

metabolites related to symbiotic associations, therefore revealing hidden biosynthetic pathways (Harwani et al., 2018; Jones and Wang, 2018; Nai and Meyer, 2018; Tan et al., 2019; Tshikantwa et al., 2018). Such alteration in the specialized metabolisms can be attributed to alteration in core metabolisms by co-culture (Cheng et al., 2013; Nguyen et al., 2019). In addition, metabolite exchanges, metabolism and/or detoxification of defence molecules (Shang et al., 2017; Vallet et al., 2017) may also occur yielding the induction of chemical novelty (Gebreselassie and Antoniewicz, 2015). Co-culture has demonstrated to significantly impact the metabolism of the involved microbial strains (Ponomarova and Patil, 2015). It can result in (a) yield enhancement, (b) *de novo* compound induction and (c) microorganisms pathways combination (Zhu et al., 2011). Nevertheless, from NPs chemist's perspective, this strategy is still random as only a few comparisons of the induced chemical diversity are available.

Up until recent years, the isolation and structural characterisation of compounds induced by co-culture were rather limited and highlighting common trends from such studies was a very complex challenge (Bertrand et al., 2014b). However, with the recent developments in analytical techniques (such as liquid chromatography coupled to high-resolution mass spectrometry), monitoring chemical induction became achievable (Bertrand et al., 2014b), leading very recently to more prolific studies exploring NPs "induction" by co-culture (Tasdemir, 2017). It is important to clearly define induction in that case; depending of the articles explored, induction can be understood as an increased in the content of a compound within the extract (which could be considered as) or the detection of a previously undetected metabolites (below detection limit when the organism is cultivated alone); both induction mechanisms are considered as up-regulation or *de novo* induction, respectively. Such induction may be the result of activation of

cryptic biosynthetic pathways (Schroeckh et al., 2009) or of the biotransformation by one of the microorganisms of compounds produced by the second one (Shang et al., 2017). In fact, even if inappropriate to describe the promotion of the formation of a novel compound, the term “induction” has been found in many articles related to co-cultures, even though the biochemical pathway activated was not described. Thus, using this term would be more in accordance with the different articles reviewed. From now in the manuscript, the define term “induction” in the fourth cases.

Some recent studies also demonstrate the possibility of co-cultivation of engineered microbial strains to access novel chemical diversity (Chen et al., 2019), or design biotechnological production strategy for known NPs (Jones and Wang, 2018; Roell et al., 2019; Wang et al., 2020). For example, Jones and colleagues have designed a specific *Escherichia coli* co-culture experiment between two specifically engineered strains for the production of flavonoids (Jones et al., 2016). While this approach is of considerable interest, from our perspective, it cannot really be considered as a strategy for the induction of microbial cryptic pathways. Furthermore, designing such stable microbial consortium for large scale production is still challenging (Stephens et al., 2019). This is particularly emphasized by the large possibility of microorganism interactions (Roell et al., 2019). Thus, currently, no generic models for biosynthetic pathway activation by co-culture exist.

In the microbial co-culture context, the metabolomics approach appears to be of great advantage to characterize the induction type (discussed in section 4). Metabolomics could be defined as a non-selective, universally applicable, comprehensive analytical approach for the identification and quantification of metabolites in a biological system (Wolfender et al., 2015). In NP research, metabolomics is considered as the large-scale analysis of metabolites of a given

organism during various physiological states which can be interpreted from a holistic manner (Sumner et al., 2003). However, only few papers have adopted this approach (Bertrand et al., 2013c; Bohni et al., 2016; Combès et al., 2012; Nguyen et al., 2019; Shen et al., 2019; Traxler et al., 2013; Wu et al., 2015; Xu et al., 2018), indicating that such strategy is not yet fully adopted in NPs chemistry.

This review covers all co-culture studies from 1994 to 2019 (to the best of our knowledge on July the 8th of 2019, Table S1). It reveals that the dawning era for the co-culture research has begun mainly from the year 2013. Thus, this corresponds to the improvement of analytical techniques, reaching their ability to accurately detect compounds within complex mixtures (Wolfender et al., 2015).

This review is an analysis of 82 articles demonstrating the induction 259 compounds *via* microbial co-culture. This review first presents the structural diversity of induced compounds based on a structural similarity clustering, followed by a discussion about observed current trends. Finally, the current challenges related to microbial co-culture experiments are highlighted to focus on some future directions that should be further explored from a NP chemist perspective.

2. Expanding the chemical diversity

The comparative analysis of chemical diversity induced through microbial co-culture is challenging and therefore, in this manuscript such diversity was explored in two different ways. First their physico-chemical properties (molecular weight and lipophilicity) were compared. Finally, structural diversity was explored constructing a structural similarity network based on "Tanimoto structure similarity index".

The comparison of physico-chemical properties of the 259 metabolites gives interesting insight into the chemical diversity of co-culture induced compounds. While molecular weight of compounds was ranging from 150 to 913 g/mol, most of them (more than 69%) were ranging from 200 to 450 g/mol (Figure 1A). This indicates that only a limited number of induced molecules corresponds to very large structures corresponding to complex biosynthesis pathways. A similar distribution was observed taking into account all reported NPs (Chen et al., 2018; Feher and Schmidt, 2003). Similarly, lipophilicity comparison indicates that AlogP ranges from -10.4 to 6.5, with about 56%, ranging from -1 to 1 (Figure 1B). AlogP represents an *in silico* evaluation of the logP value (logarithm of 1-octanol/water partition coefficient) (Ghose et al., 1998). Such medium lipophilicity (AlogP around 0) indicates rather hydrophilic molecules which possess at least reasonable water affinity to be excreted. Such property also makes them good candidates for inter-organisms' chemical communication. However, it is important to note that most of those compounds were obtained using conventional isolation strategies. Therefore, those convergent properties (close molecular weight and lipophilicity) may also reflect similar extraction and isolation procedures, in favour of the investigation of medium- and non-polar compounds, in the context of microbial co-culture. However, logP distribution centred around 0 is not observed when all NPs are taken into account, in such cases logP is centred around 3.5 (Chen et al., 2018). Thus, from a drug discovery perspective, most of the studies focusing on compounds induced during co-culture could be a good rationale to investigate future drug candidates possessing appropriate properties to be orally administered (Lipinski et al., 2012). This is a regularly observed trend observed in NP chemistry, where studies try to target isolation of the most druggable compounds (Harvey et al., 2015; Liu et al., 2017).

<<INSERT FIGURE 1 HERE>>

To explore further structural diversity induced by microbial co-cultures and therefore, highlight specific trends, a structural similarity network was constructed based on the “Tanimoto Structural Similarity Index” (see Supplementary Material, the network is also available in Cytoscape file format here <https://gitlab.univ-nantes.fr/bertrand-s-1/structural-network-of-co-culture-induced-compounds>) (Lo et al., 2016). Applying a threshold of 0.7 for the Tanimoto index clustered the 259 different structures into 9 clusters (Figure 1); such threshold being rather strict (O’Hagan et al., 2015). It is important to note that clustering of the structures based on Tanimoto Index did not always fit with biosynthetic considerations, but can reveal common structural features important for the physico-chemical properties of induced NPs which are discussed using clustering in this review. The major cluster (cluster 1) is composed of 137 molecules from “Polyketides, Macrolides and Diterpenoids” classes. All 9 clusters are discussed in the manuscript.

<<INSERT FIGURE 2 HERE>>

2.1.Non-nitrogenated induced compound clusters

2.1.1. Cluster 1 – Polyketides, Macrolides and Diterpenoids

The major cluster (cluster 1) is composed of 137 structures described in a various co-culture articles. This cluster consists of polyketides, macrolides, some diterpenoids and

miscellaneous other poly-oxygenated structures. It is composed of compounds induced *via* bacterial co-culture (n=9), fungal co-culture (n=70) or during interaction between fungi and bacteria (n=59). For the better visualization of the various chemical groups, an exploded subnetwork has been constructed using a more restrictive Tanimoto Index limit of 0.8, which is shown in Figure 3.

<<INSERT FIGURE 3 HERE>>

Arora and colleagues have isolated one new polyketide blennolide K (**1**) from the co-culture of *Setophoma terrestris* and *Bacillus amyloliquifaciens* (Arora et al., 2018). Four new polyketides, penitalarins A, B, C and nafuredin B (**2-5**) were isolated by Zhang and colleagues from a co-culture of *Penicillium variable* and *Talaromyces aculeatus* (Zhang, Z. et al., 2017), from which only nafuredin B exhibited cytotoxic activity. König and colleagues isolated two new prenylated polyketides fumicycline A and B (**6-7**) from the co-culture of *Streptomyces rapamycinicus* and *Aspergillus fumigatus* (König et al., 2013). Chagas and colleagues have characterized five polyketides stemphyperlenol, alterperyleneol, altertoxin I, alternariol and alternariol monomethyl ether (**8-12**) from the co-culture of *Alternaria tenuissima* and *Nigrospora sphaerica*. Among these, stemphyperlenol (**8**) was reported to be an antifungal compound (Chagas et al., 2013). Schroeckh and colleagues have reported four archetypal polyketides orsellinic acid, lecanoric acid, F-9775A and F-9775B (**13-16**) from the co-culture of *Streptomyces hygroscopicus* and *Aspergillus nidulans*. Among these, lecanoric acid (**14**) was found to be ATP synthesis inhibitor and F-9775A (**15**) and F-9775B (**16**) were anti-osteoporosis

agents (Schroeckh et al., 2009). It is interesting to note that lecanoric acid (**14**) was first isolated from the lichen *Pseudocyphellaria crocata* (Maass, 1975) and also observed in many of such symbiotic organism (Ahmann and Mathey, 1967; Luo et al., 2009; van Herk and Aptroot, 2007). Interestingly, another orsellinic acid derivatives was isolated by Bertrand and colleagues and characterized as hydroxysulfoxy-2,2''-dimethylthielavin P (**17**) from the co-culture of *Trichophyton rubrum* and *Bionectria ochroleuca* (Bertrand et al., 2013c). Wang and colleagues isolated four phenolic polyketides alternariol, (9R, 14S)-epoxy-11-deoxyfunicone, deoxyfunicone and vermistatin (**11,18-20**) from the co-culture of *Streptomyces fradiae* 007 and *Penicillium* sp. WC-29-5, from which, (9R, 14S)-epoxy-11-deoxyfunicone (**18**) was found to be a previously unreported structure with moderate cytotoxic activity (Wang et al., 2014). Other similar phenolic polyketides lateropyrone (**21**), dihydrolateropyrone (**22**), fusatricinone A, B, C, D (**23-26**) were isolated from the co-culture of *Fusarium tricinctum* and *Streptomyces lividans* (Moussa et al., 2019). Other polyketides were also isolated from this latter co-culture, macrocarpon C (**27**), (–)-citreoisocoumarin (**28**), zearalenone (**29**), 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (**30**, also named Aloesol). Among those induced compounds lateropyrone (**21**) and zearaleone (**29**) exhibited antibiotic activity (Kosawang et al., 2014; Ola et al., 2013), zearalenone (**29**) being a known mycotoxin with chronic estrogenic effects on mammals (Hueza et al., 2014; Kuiper-Goodman et al., 1987). Macrocarpon C (**27**) and (–)-citreoisocoumarin (**28**) were also isolated from the co-culture of *F. tricinctum* with the other bacteria *Bacillus subtilis* along with another isocoumarin derivative (–)-citreoisocoumarinol (**31**) (Ola et al., 2013). Another phenolic polyketide bikaverin (**32**) was highlighted by Maldi-MS imaging to only detected in the confrontation zone of *Fusarium fujikuroi* and *Ralstonia solanacearum* (Spraker et al., 2018).

Gordonic acid (**33**), which is an acyclic polyene polyketide, demonstrated weak antibacterial activity (Park et al., 2017). Resistomycin (**34**) and 2,5,6-trihydroxy-4,6-diphenylcyclohex-4-ene-1,3-dione (**35**) exhibited cytotoxic activity and correspond to pentacyclic or phenyl polyketide, respectively (Carlson et al., 2014; Xu et al., 2018). Other polyketides janthinopolyenemycin A and B (**36, 37**), possessing moderate anti-*candida* activity, were also reported to be induced during the co-culture of two strains of *Janthinobacterium* spp. (ZZ145 and ZZ148) (Anjum et al., 2018). Five perylene quinonoid polyketides were induced during the co-culture of *Shiraia* sp. S9 and *Pseudomonas fulva* SB1. These correspond to the pigments hypocrellin A, C (**38, 39**) and elsinochrome A, B, C (**40-42**) which were evaluated as photosensitizers for photodynamic therapy (Ma et al., 2019).

This cluster was also composed of eight 16-atoms ring macrolides berkeleylactone A, B, C, D, E, F, G, H (**43-50**) isolated by Stierle and colleagues from the co-culture of *Penicillium fuscum* and *Penicillium camembertii/clavigerum*; three of them (**43, 45, 48**) exhibited cytotoxic activity (Stierle et al., 2017).

In addition, terpenes were also reported in this cluster. Cho and Kim have reported antifouling diterpene lobocompactol (**51**) from the co-culture of *Streptomyces cinnabarinus* and *Alteromonas* sp. (Cho and Kim, 2012). Oh and colleagues have reported the induction of four cytotoxic pimarane diterpenoids libertellenone A, B, C and D (**52-55**) from the co-culture of an α -proteobacterium (strain CNJ-328) and *Libertella* sp. (Oh et al., 2005). Zhang and colleagues isolated two sesquiterpenes microsphaeropsisins B (**56**) and C (**57**) by co-cultivation of *Trichoderma* sp. (Strain 307) with *Acinetobacter johnsonii* (Strain B2) (Zhang, L. et al., 2017). Microsphaeropsisin C (**57**) was having moderate α -glucosidase inhibitory activity. Other sesquiterpenes melledonal A, C (**58, 59**) were induced by the co-culture of *Gloeophyllum*

abietinum and *Heterobasidion annosum* (Sonnenbichler et al., 1994), and isocaryophyllene, α -humulene, cyclocaryophyllan-4-ol (**60-62**) by the co-culture of *Fusarium oxysporum* with *Serratia* sp. and *Achromobacter* sp. (Minerdi et al., 2009). Interestingly, those latter three compounds are volatile substances and represent rare examples of induction highlighted by gas chromatography coupled to MS. Zhou and colleagues isolated five new sesquiterpenes, nigirpexin A, B, C, D and nigrosirpexin A (**63-67**) from the co-culture of *Nigrospora oryzae* with *Irpex lacteus* (Zhou et al., 2018). All these latter compounds exhibited antifungal activity, nigrosirpexin A (**67**) being also an inhibitor of acetylcholinesterase.

The meroterpenoid austin (**68**) was reported to be induced during the co-culture of *Talaromyces purpurogenus* H4 and *Phanerochaete* sp. H2 and possesses moderate trypanocidal activity (do Nascimento et al., 2019).

Various citrinin analogues were reported to be induced during co-culture. Citrifelin A, B (**69-70**) were from the co-culture of the fungi *Penicillium citrinum* and *Beauveria felina* (Meng et al., 2015). Recently, Yang and colleagues reported the induction of citrinin (**71**) along with 10 other analogues: seco-penicitrinol A (**72**), penicitrinol A and L (**73, 74**), penicitrinone A and F (**75, 76**), dihydrocitrinone (**77**) and decarboxydihydrocitrinone (**78**) during the interaction of *Penicillium citrinum* and *Aspergillus sydowii* (Yang et al., 2018). During this latter co-culture experiment, two other phenols were induced, called phenol A (**79**) and phenol A acid (**80**). From all those citrinin analogues only moderate antimicrobial activity was observed (Meng et al., 2015; Yang et al., 2018).

Various other compounds exhibiting at least moderate antimicrobial activity are associated with this cluster, such as actinorhodin (**81**) (Mavituna et al., 2016; Traxler et al., 2013), BE-31405 (**82**) (Nonaka et al., 2015), the mycoalexin *O*-methylnellein (**83**) (Glauser et al., 2009),

the xanthone derivative 8-hydroxy-3-methyl-9-oxo-9*H*-xanthene-1-carboxylic acid methyl ether (84) (Li et al., 2011), the benzaldehyde derivative ethyl 5-ethoxy-2-formyl-3-hydroxy-4-methylbenzoate (85) (Wang, J. et al., 2013) and the cytotoxic benzophenone pestalone (86) (Cueto et al., 2001). 5-Formylsalicylic acid (87) (Keilhofer et al., 2018) was found to be a potential virulence factor of fungal plant pathogens. Three γ -butyrolactones chojalactone A, B, C (88-90) with moderate cytotoxicity were reported to be induced in co-culture of *Streptomyces* sp. CJ5 and *Tsukamurella pulmonis* TP-B0596 (Hoshino et al., 2015b). Phytopathogen dependent NPs harzianolide (91), sorbicillinol (92) and T39 butenolide (93) were isolated from the co-culture of *Moniliophthora roreri* and *Trichoderma harzianum* (Tata et al., 2015). Interestingly, harzianolide (91) is able to promote plant growth in tomato models (Cai et al., 2013). Similarly, other butenolide (γ -butyrolactones) chaetobutenolides A, B, C and WF-3681 methyl ester (94-97) were isolated from the co-culture of *Chaetomium* sp. with *Pseudomonas aeruginosa* (Ancheeva et al., 2017). Similarly, small butyrolactones isobutyrolactone II (98), 4-*O*-demethylisobutyrolactone II (99) were isolated from the interactions of *Aspergillus terreus* with *Bacillus* sp. strains (Chen et al., 2015). It is important to pinpoint this particular induction of butyrolactones as they are well known compounds involved in interspecies communication, especially in quorum-sensing mechanisms in bacteria (Niu et al., 2016).

Other compounds possessing polar head along with an alkyl side chain are also present in this cluster, such compounds may be expected to also be involved in interspecies communication, even though no information is available. Penixylarins A, B, C, D (100-103), 1,3-dihydroxy-5-(12-hydroxyheptadecyl)benzene (104) and 1,3-dihydroxy-5-(12-sulfoxyheptadecyl)benzene (104) were induced during the co-culture of *Penicillium crustosum* PRB-2 and *Xylaria* sp. HDN13-249 (Yu, G. et al., 2019). Among those compounds, penixylarins

B, C (**101**, **102**), 1,3-dihydroxy-5-(12-hydroxyheptadecyl)benzene (**104**) and 1,3-dihydroxy-5-(12-sulfoxyheptadecyl)benzene (**105**) exhibit moderate antibacterial activity and penixylarin C (**102**) potential antituberculosis effect. Two other compounds possess a similar scaffold: phexandiol A, B (**106-107**). They are produced during the interaction between *Phoma* sp. YUD17001 and *Armillaria* sp. 2TG-2016, however, no antimicrobial nor cytotoxic activities were observed (Li et al., 2019). Another example lies in the induction of the pyran derivative chaunopyran A (**108**) during the co-culture of *Chaunopycnis* sp. CMB-MF028 and *Trichoderma hamatum* CMB-MF030 (Shang et al., 2017). An unusual dimer of such scaffold is also induced during the interaction between *Penicillium pinophilum* FKI-5653 and *Trichoderma harzianum* FKI-5655, it corresponds to stromemycin (**109**) (Nonaka et al., 2011). This interaction also yields the induction of secopenicillide C (**110**), penicillide (**111**, also called Vermixocin A) and MC-141 (**112**).

Five azaphilones nigbeauvin A, B, C, D, E (**113-117**) were isolated by Zhang and colleagues (Zhang et al., 2018). Nigbeauvin A (**113**) has nitric oxide (NO) inhibitory activity and antibacterial activity, while nigbeauvin B (**114**) has only NO inhibitory activity. (3R)-5-oxo-de-O-methylasiodiplodin (**118**) and (3R, 7R)-7-hydroxy-de-O-methylasiodiplodin (**119**) were reported, from the co-culture of a *Trichoderma* sp. strain with *Acinetobacter johnsonii*, to have the α -glucosidase inhibitory activity (Zhang, L. et al., 2017). Akone and colleagues isolated six compounds: shikimeran A, bipherin A, chorismeron, serkydayn, isosulochrin, protocatechuic acid methyl ester (**120-125**) from the co-culture of *Chaetomium* sp. with bacteria (Akone et al., 2016). Serkydayn (**123**) has moderate antibacterial activity and cytotoxic activity.

It is important to emphasise the case of the induction of the triterpene ergosterol (**126**) from the co-culture of two *Aspergillus* sp. strains (Zhu et al., 2011). This compound is known to have

a key role in fungal membrane integrity (Abe and Hiraki, 2009) and also possesses antiproliferative activity on cancer cells (Li et al., 2015; Ory et al., 2019).

In the co-culture studies, some compounds were reported without activities such as (E)-2-(3-hydroxyprop-1-en-1-yl)-phenol (**127**) (Wu et al., 2015), the furanone derivatives sclerotiorumin A, B (**128**, **129**) (Bao et al., 2017), the coumarin derivative 7-(γ,γ -dimethylallyloxy)-6-hydroxy-4-methylcoumarin (**130**) (Wang et al., 2015), the shikimic acid derivatives chaetochorismin (**131**), shikimeran B (**132**) (Ancheeva et al., 2017), the chromone 7-methoxy-2,3-dimethylchromone-4-one (**133**) (Yu, M. et al., 2019) and the sesquiterpenoid naphthoquinone debromomarinone (**134**) (Khalil et al., 2019).

Additional compounds (2E,4E)-3-(2-carboxy-1-hydroxyethyl)-2,4-hexadienedioic acid (**135**) (Wu et al., 2015), oosponol (**136**), oospoglycol (**137**) (Sonnenbichler et al., 1994) were also attached to this network.

2.1.2. Cluster 2 – Linear polyketides, Oxylipins and Fatty acids

The second cluster (Cluster 2) is composed of 16 compounds reported in seven different articles, corresponding to linear polyketides, oxylipins and fatty acids (Figure 2).

A large variety of microorganisms have shown to specifically produce linear polyketides, fatty acids and their oxidation products oxylipins in response to mixed cultures. Kossuga and colleagues have reported two linear polyketides (Z) 2-ethylhex-2-enedioic acid (**138**) and (E) 4-oxo-2-propylideneoct-7-enoic acid (**139**) from the co-culture of two marine-derived *Penicillium* sp. Ma(M3)V and *Trichoderma* sp. Gc(M2)1. Wrede and colleagues generated an efficient system for lipid production, harvesting micro-algal cells and waste water treatment by co-cultivation of *Aspergillus fumigatus* and microalgae. Their co-cultivation demonstrated

synergistic and additive effects on lipid yield, wastewater bioremediation efficiency and biomass production. The co-culture produced palmitic acid, stearic acid, oleic acid and linoleic acid (**140-143**), which can be developed as a renewable source of biofuels (Wrede et al., 2014). Do Nascimento and colleagues studied the effect of co-culture of bacteria on the growth promotion of microalgae (Do Nascimento et al., 2013). They characterized an ω 3 fatty acid, stearidonic acid (**144**) from the co-culture of *Rhizobium* strain 10II and *Ankistrodesmus* sp. strain SP2-15. Combès and colleagues investigated the co-culture potential of phytopathogen *Fusarium oxysporum* and endophytic fungus *Paraconiothyrium variabile* for the production of oxylipins 13-oxo-9,11-octadecadienoic acid (**145**), 13-hydroperoxy-9,11-octadecadienoic acid (**146**) and reported their role in the regulation of development processes and also in the mycotoxin beauvericin production regulation in *F. oxysporum* (Combès et al., 2012). It is interesting to note that such oxylipins related compounds are regularly linked to interspecies communication (Wasternack and Feussner, 2018) as it was the case for butyrolactones from Cluster 1.

A series of polyhydroxylated fatty acids and esters: phomester A, B, (E)-5,9-dihydroxydodec-6-enoic acid (**147-149**), were isolated from the co-culture of *Phoma* sp. YUD17001 and *Armillaria* sp. 2TG-2016 (Li et al., 2019). Similarly, polyhydroxylated alkyl chain were also induced during the co-culture of *Trametes robiniophila* Murr and *Pleurotus ostreatus*. These correspond to postrediene A, B, C (**150-152**) and were not reported with any promising activities (Shen et al., 2019).

Furthermore, the tricarboxylic fatty acid bongkreic acid (**153**) was also produced by the bacteria *Burkholderia gladioli* when co-cultured with the fungus *Rhizopus microsporus*. Interestingly, this highly toxic compound possesses fungal growth inhibition properties, leading to assume its putative role in the co-culture (Ross et al., 2014).

2.2. Non-alkaloidic nitrogenated induced compound clusters

2.2.1. Cluster 3 – Cyclic peptides, diketopiperazines and related compounds

This cluster 3 consists of 60 compounds from 18 different co-culture articles (Figure 2).

Guo and colleagues isolated pseudoxylallemycin A-F (**154-159**), cyclized tyrosine-containing non-ribosomal tetrapeptides, from the co-culture extract of *Pseudoxylaria* sp. and *Microtermes* sp. Pseudoxylallemycin A, B, C, D (**154-157**) were reported to possess antimicrobial activity, antiproliferative and cytotoxic properties, while pseudoxylallemycin E and F (**158, 159**) were inactive (Guo et al., 2016). Other cyclic polypeptides were also observed to be induced and possess antimicrobial activity. Cyclo-(L-leucyl-trans-4-hydroxy-L-prolyl-D-leucyl-trans-4-hydroxy-L-proline) (**160**), cyclo-(D-proline-L-tyrosine-L-proline-L-tyrosine) (**161**) and cyclo-(glycine-L-phenylalanine-L-proline-L-tyrosine) (**162**) were observed in the co-culture of *Phomopsis* sp. K38 and *Alternaria* sp. E33 (Huang et al., 2014; Li et al., 2014). The mixed fermentation of *Umezawaea* sp. RD066910 and *Tsukamurella pulmonis* TP-B0596 yielded the production of umezawamide A, B (**163, 164**) (Hoshino et al., 2018b), of *Emericella* sp. and *Salinispora arenicola* provided emericellamides A, B (**165, 166**) (Oh et al., 2007), *Fusarium tricinctum* with *Streptomyces lividans* or *Bacillus subtilis* yielded fusaristatin A (**167**) (Moussa et al., 2019; Ola et al., 2013), and *Aspergillus flavipes* and *Streptomyces* sp. activate the production of rosellichalasin (**168**), aspochalasin E, P, H, M (**169-172**) and 19,20-dihydro-aspochalasin D (**173**) (Yu et al., 2016). Finally, the co-culture of unidentified fungi induces the production of (–)-byssochlamic acid imide (**174**) (Ding et al., 2017).

Various diketopiperazines were induced during co-culture. Khalil and colleagues reported the induction of the bacteriostatic cyclo-(L-phenylalanine-trans-4-hydroxy-L-proline) (**175**)

377 during the co-culture of *Streptomyces* sp. and *Aspergillus* sp. (Khalil et al., 2019). Rateb and
378 colleagues isolated antiparasitic diketopiperazines brevianamide F (**176**), spirotryprostatin A
379 (**177**), 6-methoxy spirotryprostatin B (**178**), fumitremorgin B, C (**179**, **180**), 12,13-dihydroxy-
380 fumitremorgin C (**181**), verruculogen (**182**) from the co-culture of *Streptomyces bullii* and
381 *Aspergillus fumigatus* (Rateb et al., 2013). From this co-culture they also reported the induction
382 of 11-*O*-methylnpseurotin A (**183**) and 11-*O*-methylnpseurotin A₂ (**184**); this latter compound
383 exhibiting antiparasitic activity. Other diketopiperazines notoamide C, D, F, M, Q (**185-189**), (–
384)-notoamide B (**190**), 17-*epi*-notoamide Q (**191**), dehydronotoamide C (**192**), 17-
385 hydroxynotoamide D (**193**), 17-*O*-ethylnotoamide M (**194**), 10-*O*-ethylnotoamide R (**195**),
386 sclerotiamide (**196**), 10-*O*-ethylsclerotiamide (**197**), 10-*O*-acetylsclerotiamide (**198**) were
387 produced during the interaction between *Aspergillus sulphureus* and *Isaria felina* (Afiyatulloev et
388 al., 2018).

389 In addition, aspergicin (**199**) from the co-culture of two *Aspergillus* sp. strains (Zhu et al.,
390 2011), *N*-demethylophiosetin (**200**), pallidorosetin A, B (**201**, **202**) from the co-culture of
391 *Saccharopolyspora erythraea* and *Fusarium pallidroseum* (Whitt et al., 2014) and niizalactam
392 A, B, C (**203-205**) from the co-culture of *Streptomyces* sp. NZ-6 and *Tsukamurella pulmonis* TP-
393 B0596 (Hoshino et al., 2015a) were reported as induced.

394 Five nitrogenated compounds were distantly attached to this cluster. Among all compounds
395 from this cluster, one was reported in two different articles. The pyrrol derivative
396 undecylprodigiosin (**206**) was reported induced in two bacterial mixed fermentation involving
397 *Streptomyces Coelicolor* with either *E. coli* (Mavituna et al., 2016) or *Corallococcus coralloides*
398 (Schäberle et al., 2013). Prodiginine (**207**), a very similar compound was induced during the
399 interaction of *Streptomyces coelicolor* and actinomycete strain (Traxler et al., 2013). Those red

pigments prodiginins are known to exhibit a wide range of activity: immunosuppressive, anticancer and antibacterial (Hu et al., 2016). It was demonstrated that such compounds could be up-regulated by extracellular cell–cell signaling (quorum sensing) due to the presence of γ -butyrolactones (Williamson et al., 2006). Additionally, the aminoglycosides rhodostreptomycin A, B (**208**, **209**) were reported from the mixed fermentation of *Rhodococcus fascians* and *Streptomyces padanus* (Kurosawa et al., 2008). Moreover, complex nitrogenated fatty acids were also attached to this network. They correspond to borrelidin J and K (**210**, **211**) produced during the interaction between *Streptomyces rochei* MB037 and *Rhinocycladiella similis* 35 (Yu, M. et al., 2019). Interestingly, borrelidin J (**210**) possesses antibacterial activity against methicillin-resistant *Staphylococcus aureus* at a low μ M range. Additional compounds heronapyrrole B (**212**) (Khalil et al., 2019) and phomester C (**213**) (Li et al., 2019) was also attached to this network. Finally, the induction of taxol (**214**) from the co-culture of *Paraconiothyrium* SSM001 with other fungal strains (Soliman and Raizada, 2013) is particularly interesting. This corresponds to the “re-discovery” of a very well-known anticancer drug first isolated from *Taxus baccata*, which in fact was produced by endophytic fungi instead of the plant itself.

2.2.2. Cluster 4 – Other non-ribosomal peptides

This cluster 4 consists of 5 peptides (Figure 2), either linear tetrapeptides or cyclic hexapeptides from two different co-culture articles. Wang and colleagues isolated depsipeptides subenniatin A, B (**215**, **216**) from the co-culture of *Fusarium tricinctum* and *Fusarium begonia* (Wang, J.-p. et al., 2013). In addition, enniatin A1, B, B1 (**217-219**) induced during the co-culture of *Fusarium tricinctum* with *Streptomyces lividans* or *Bacillus subtilis* (Moussa et al., 2019; Ola et al., 2013) was present in this cluster.

2.2.3. Clusters 5 and 6 – Miscellaneous Non-alkaloidic nitrogenated compound

Two small clusters contain 16 compounds with at least one nitrogenated function. Those two clusters are less organized based on chemical structures of compounds as many are distantly attached together (Figure 2). This explains inconsistency of the clustered structures.

The first cluster is composed of 2-(carboxymethylamino) benzoic acid (**220**) isolated from the co-culture of *Fusarium tricinctum* and *Bacillus subtilis* (Ola et al., 2013) and bionectriamine A, B (**221**, **222**) isolated from the co-culture of a *Bionectria* sp. strain with bacteria (Kamdem et al., 2018). In additions enacyloxin IIa, iso enacyloxin IIIa (**223**, **224**) where reported from the interaction between *Burkholderia gladioli* and *Rhizopus microsporus* (Ross et al., 2014); only iso enacyloxin IIIa (**224**) exhibits antimicrobial activity.

The second cluster contains two compounds known to act as siderophores: desferrioxamine B (**225**) and coelichelin (**226**) induced during the co-culture of *Streptomyces coelicolor* and actinomycete (Traxler et al., 2013), thus responsible for iron scavenging in the environment and then supporting microbial growth (Bertrand et al., 2013a; Hider and Kong, 2010). The mixed fermentation of *Catenuloplanes* sp. RD067331 and *T. Pulmonis* yielded the production of catenulobactin A, B (**227**, **228**) possessing iron-chelating and moderate cytotoxicity activity (Hoshino et al., 2018a). The peptides dentigerumycin E (**229**) exhibiting moderate antitumor activity was reported as induced during the co-fermentation of *Streptomyces* sp. strain JB5 and *Bacillus* sp. (GN1) (Shin et al., 2018). Two additional compounds were reported alchivemycin A (**230**) and 11S-hydroxy-1-methoxyfusaricide (**231**) (Li et al., 2017) from the co-culture of *Tsukamurella pulmonis* with *Streptomyces endus* S-522 (Onaka et al., 2011) and of *Camporesia sambuci* FT1061 with *Epicoccum sorghinum* FT1062, respectively. This cluster is also composed of pyrazine alkaloids hydroxyaspergillin, neoaspergillic acid (**232**, **233**) possessing cytotoxic and antibacterial activity respectively (Bao et al., 2017; Zhu et al., 2011). In addition,

an oxadiazine derivative sclerotiorumin C (**234**) issued from the combined culture of *Aspergillus sclerotiorum* and *Penicillium citrinum* (Bao et al., 2017) was also distantly associated with this cluster. Finally, one thiodiketopiperazine glionitrin A (**235**) was also present in this cluster. It was induced during the mixed fermentation of *Aspergillus fumigatus* with *Sphingomonas* sp., this compound exhibiting antimicrobial activity and cytotoxicity (Park et al., 2009).

2.3. Alkaloidic induced compound clusters

2.3.1. Cluster 7 – Benzylamine Alkaloids

A small cluster (Cluster 7) was observed covering nine structures from three different studies (Figure 2). Three structures were indolocarbazole alkaloids, a chemical family often isolated from actinomycetes (Cañedo Hernandez et al., 2000; Fu et al., 2012; Zhang et al., 2012). Hoshino and colleagues isolated a indolocarbazole alkaloids, arcyriaflavin A and E (**236**, **237**), together with BE-13793C (**238**) from the co-culture of *Streptomyces cinnamoneus* NBRC 13823 and a mycolic acid-containing bacteria *Tsukamurella pulmonis* (Hoshino et al., 2015c); compounds **237** and **238** exhibiting moderate cytotoxic activity on P388 murine leukaemia cells. Feng and colleagues isolated marinamide (**239**) and its methyl ester (**240**) from the co-culture of two mangrove endophytic fungi (strain Nos. 3893 and 1924) (Zhu and Lin, 2006). One more compound, isolated from co-culture of the two sponge-associated actinomycetes *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163, is also associated with this cluster: 5a,6,11a,12-tetrahydro-5a,11a-dimethyl[1,4]benzoxazino[3,2-*b*][1,4]benzoxazine (**241**) (Dashti et al., 2014).

Two additional compounds were distantly attached to this cluster: austramide (**242**) from the co-culture of *Aspergillus austroafricanus* with either *Bacillus subtilis* or *Streptomyces*

lividans (Ebrahim et al., 2016) and 1,6-dihydroxyphenazine (**243**) from the co-culture of *Actinokineospora* sp. EG49 with *Nocardiopsis* sp. RV163 (Dashti et al., 2014).

2.3.2. Cluster 8 – Phenazine alkaloids

This cluster 8 represents height phenazine compounds reported in three co-culture articles (Figure 2). Angell and colleagues isolated pyocyanin (**244**), which was overproduced in the co-culture of *Enterobacter* sp. Pup14A and *P. aeruginosa*, a well-known producer of such type of compounds (Angell et al., 2006). Pyocyanin (**244**) is reported to possess antimicrobial and cytotoxic activity (Jayaseelan et al., 2014). Such compounds are known for their toxic behaviour against many organisms. Therefore, their induction during micro-organisms co-cultures can be considered as a mechanism of defence of the bacteria. Interestingly, many fungi like *Aspergilli* possess specific detoxification mechanisms through phenazine specific biotransformation. Considering this, Moree and colleagues studied the production of different phenazines during the co-culture of *P. aeruginosa* and *A. fumigatus*, which led to the observation of the induction of some compounds and/or the appearance of biotransformed products, such as pyocyanin (**244**), phenazine-1-carboxamide (**245**), 1-hydroxyphenazine (**246**), phenazine-1-carboxylic acid (**247**), 1-methoxyphenazine (**248**), phenazine-1-sulfate (**249**) and 5-*N*-methylated phenazine-1-carboxylic acid (**250**) (Moree et al., 2012). In this study, differences were clear between the “induction mechanisms” corresponding to biosynthesis activation and biotransformation. It is important to note that phenazines correspond to a class of compounds exhibiting a large variety of biological activity (Laursen and Nielsen, 2004). Dashti and colleagues isolated antibacterial and antiparasitic *N*-(2-hydroxyphenyl)-acetamide (**251**) from the co-culture of two actinomycetes *Actinokineospora* sp. EG49 and *Nocardiopsis* sp. RV163 (Dashti et al., 2014).

Fusaric acid (**252**) induced during the interaction between *Fusarium oxysporum* and *Sarocladium strictum* (Bohni et al., 2016) and quinomeran (**253**) from the co-culture of *Chaetomium* sp. with bacteria (Akone et al., 2016) were also present in this network.

2.3.3. Cluster 9 – Tryptamine and Pyrrole alkaloids

One small cluster (Cluster 9) consisting of six compounds corresponded to two different articles (Figure 2). Yu and colleagues isolated and characterized algicidal *N*-acetyltryptamine (**254**), *N*-propanoyltryptamine (**255**), bacillamide A, B, C (**256-258**) from the liquid fermentation of *Streptomyces* sp. and *Bacillus mycoides* (Yu et al., 2015). Among all those tryptamine derivatives, only bacillamide A (**256**) exhibits algicidal activity (Wang et al., 2017). Bao and colleagues isolated 1-(4-benzyl-1H-pyrrol-3-yl)ethanone (**259**) from the liquid fermentation of *Aspergillus sclerotiorum* and *Penicillium citrinum* (Bao et al., 2017).

3. Current trends in co-culture induction

In terms of culture methods, out of 82 studies, 24 papers used solid medium, 59 studies used liquid fermentation (also called mixed fermentation). Among those, two studies used both strategies (Chagas et al., 2013; Sonnenbichler et al., 1989). Mapping such culture methods on the network (Fig. S1), it remains obvious that no clear relationship could be observed between induced structures and the culture method with two exceptions. The cluster 2 “linear polyketides, Oxylipins and Fatty acids” only contains compounds reported from liquid fermentation. The molecules present in cluster 3 “Cyclic peptides, diketopiperazines and related compounds” are also mostly obtained by liquid fermentation. Other cluster specific culture condition observed are too small and represent a too low number of articles to be able to give more general

consideration. Therefore, unless studies aim at inducing structures closely related to cluster 2 and 3, both co-culture conditions should be evaluated.

Regarding microorganisms' combination, researchers have adopted different strategies. Microorganisms were chosen from a common ecological niche to reveal microbial community chemical interactions (Kuhlish and Pohnert, 2015). Alternatively, sometimes one considers that ecologically irrelevant interactions could stress the organisms even more (Bertrand et al., 2013b). Based on structural considerations, many teams had worked on inter-kingdom microbial interactions (*i.e.*, bacteria and fungus) to delineate the biological origin of *de novo*-induced isolated compounds based on biosynthetic differences (some key skeletons being only produced by specific kingdoms related biosynthetic pathways) (Ola et al., 2013).

The articles reporting compound inductions were compared based on the micro-organisms grown together (Figure 4). Interestingly, most of those microbial co-cultures were involving fungi (65 articles) followed by bacteria (47 articles). Only very few articles were using micro-algae (1 articles) which may be due to more difficult culture conditions. In addition, most articles (54 articles) reported kingdom specific co-cultures (fungus *vs.* fungus or bacteria *vs.* bacteria). Such observation may be mainly due to laboratory expertise in only one type of microorganisms. Thus, it is interesting to note that fungal and bacterial co-culture are mostly exploited as case studies for compound induction. Mapping such involved kingdom on the network (Fig. S2) obviously shows that, unfortunately, no relationship could be observed between induced structure and the organism kingdoms. Thus, up to the current knowledge, the reported structural diversity of induced compounds is still limited and more diversity is expected to be published in the near future.

<<INSERT FIGURE 4 HERE>>

In addition to co-culture involving bacteria and/or fungi, only very few studies explore other microbial sources. For example, only one used microalgae as indicated before (Wrede et al., 2014). However, in the near future, one can imagine to perform co-culture experiments with other microorganisms like amoeba (Zarei et al., 2019), or with plant, human and/or animal cells (Hara et al., 2018). Taking into account such possibilities, an improved chemical diversity should be accessible.

Finally, it remains important to highlight biological activity evaluated on the isolated compounds. However, it is important to note that in most studies, compounds were isolated for the first time in the studied microbial co-culture. Thus in that case, the biological evaluation was only reported in the article itself. This makes difficult to get a clear view of which biological activity to expect from such induced compounds. Interestingly, more than half of the compounds were reported without any activity (139 compounds), this corresponds to absence of biological evaluation but also to absence of biological activity (as many times, unfortunately, low activity values are not mentioned). Like in most NP studies, in the case of co-culture induced compounds, relevant biological activity reported were mostly antibacterial (37%), cytotoxic (35%) and antifungal (7%) (Figure 5 and figure S3 if moderate activity are taken into consideration). However, the proportion of antimicrobial activity represents 44% of the evaluated activity, and is largely, the most evaluated activity. This clearly indicates that NP chemists mostly expect to discover such activity of such induced compounds (Lodhi et al., 2018;

Netzker et al., 2018; Zhang and Straight, 2019). It is interesting to note other biological activity that could be related to interspecies interaction, with the presence of antifouling (Cho and Kim, 2012), algicidal (Wang et al., 2017), phytotoxic compounds (Glauser et al., 2009), siderophores (Traxler et al., 2013) and plant growth promoters (Cai et al., 2013; Tata et al., 2015). But, yet, it remains unclear if this trend is real or an artefact of rationality, as this evaluation seems to be the first choice of investigators.

<<INSERT FIGURE 5 HERE>>

4. Challenges of Co-Culture Strategies for Chemical Diversity Enhancement

The use of co-culture for induction of NPs is a very challenging process and various difficulties appears to all researchers working in this area. The challenges encountered from NP Chemist's point of view are: (1) how to observe the metabolic induction in co-culture? (2) how reproducible are such inductions? (3) what is the appropriate time to observe the induction? (4) are compound isolation and identification a challenging task?

These challenges are explained in detail as follow.

4.1.How to observe the metabolic induction in co-culture?

One of the main challenge in co-culture chemical analysis, remains the monitoring of induction itself as well as chemical diversity evaluation. This is why, even if co-culture

experiments were already tested in the mid 80's (Tokimoto et al., 1987), a much larger number of articles have started to be published in the last ten years as better accurate and sensitive analytical techniques in NP research studies occurred (Bertrand et al., 2014b; Wolfender et al., 2019).

In the case of microbial co-culture, historically chemical induction was demonstrated using targeted strategy on known compounds (Tokimoto et al., 1987) using HPLC-UV detection for example. Nowadays, chemical profiling is mostly achieved by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) or mass spectrometry imaging (MS-imaging) (Bertrand et al., 2014b). In the reported cases, NMR is mostly used to reveal core metabolism alterations, however providing information mostly about core metabolome alterations (Cheng et al., 2013; Martínez-Buitrago et al., 2019; Nguyen et al., 2019). Very recently, a 2D NMR fingerprint strategy (Buedenbender et al., 2018) has been reported to highlight metabolic alteration thus indicating the induction of polyketides, peptides and aromatic compounds base on specific observed resonances (Buedenbender et al., 2019). Untargeted approaches were proven to be more efficient, in particular when metabolomic strategies were employed. These latter strategies, by profiling various biological replicates, generate large datasets and thus need specific chemometric analyses to delineate chemical modifications attributed to each organism (Bertrand et al., 2014b; Geurts et al., 2017; Jansen et al., 2015; Wang and Bertrand, 2019). In this context, defining what is induction is already a complex question. It could be considered as “*de novo* induction” (not detected in pure strain culture) or “up-regulated” (detected at lower concentration in pure strain culture). Unfortunately, such a distinction has to be considered in regards to the analytical techniques used to highlight such induction. It is clear that detection limits for thin layer chromatography (TLC), HPLC-UV or UHPLC-MS are different. Therefore,

it remains complicated to conclude on general induction pattern when studies using TLC (Li et al., 2019) are compared with HPLC (Kossuga et al., 2013; Ma et al., 2019; Nonaka et al., 2015) or metabolomic driven LC-MS (Bertrand et al., 2013c; Bohni et al., 2016; Combès et al., 2012; Shen et al., 2019; Wu et al., 2015; Xu et al., 2018). However, in the future studies (or publication), the type in induction observed should be important to mention. This should further help to discuss chemical diversity in regards to induction type.

Alternatively, approaches based on MS-imaging were also successfully applied to co-culture, providing information about the spatial metabolite distribution and metabolisation (Moree et al., 2013; Tata et al., 2015). This alternative approach highlights metabolite located specifically in the microorganisms' interaction zone. It provides an interesting insight into microbial interaction mechanisms. Interestingly, both strategies are complementary in revealing the very complex nature of such co-culture induction.

4.2.How reproducible are such inductions?

Intriguingly, only very few examples exist of in depth studies of compound induction mechanisms by co-culture. In addition, only rare cases report the reproduction of co-culture experiments. To the author knowledge, only the interaction of *B. obtusa* and *E. lata* was attempted two times highlighting similar induction pattern (Azzollini et al., 2018; Glauser et al., 2009). In addition, some articles report the use of closely related organisms with the induction of identical compounds, such as *F. tricinctum* with different bacteria (*S. lividans* or *B. subtilis*) yielded macrocarpon C (**27**), (–)-citreisocoumarin (**28**), fusaristatin A (**168**), enniatin A1, B and B1 (**217-219**) induction (Moussa et al., 2019; Ola et al., 2013).

One reason may be the focus of NP-chemists on discovering new chemical entities, which yield to model change once structures are finally published. However, a second reason lays in the difficulties encountered when reproducing a co-culture experiment is attempted. As many parameters should be taken into consideration for microbial growth, some of them could be complicated to fully control, such as the history of inoculum for the producing cultures (number of pre-culture and/or pre-culture treatment) (Imhoff, 2016). This may explain such difficulties and thus the low number reproduced studies.

Unfortunately, to the best of our knowledge, no studies report difficulties to reproduce previous experiments in order to study in-depth microbial interaction mechanisms or optimisation of co-culture conditions for compound production.

4.3. What is the appropriate time to observe the induction?

The NP biosynthesis is a very dynamic process, and recent studies have revealed that specialized metabolites may be produced at every stage of microbial growth (Roullier et al., 2016). Thus, in the context of microbial co-culture, such study conditions are much more complex. Unfortunately, only few studies exist on the impact of time on co-culture induction (Arora et al., 2019; Azzollini et al., 2018; Bertrand et al., 2014a). For example, the study of *Aspergillus sclerotiorum* and *Xylaria psidii* shows antimicrobial activity induction only at a specific culture time (Arora et al., 2019). Similarly, the metabolic study through time of a *Fusarium* sp. strain with *A. clavatus* Sin141 clearly shows the dynamic nature of compound induction, with some of them being induced only at specific growth time (Bertrand et al., 2014a; Geurts et al., 2017).

Therefore, to get an exhaustive view of compound induction in a specific co-culture experiment, it remains crucial to use sensitive analytical techniques and to explore the influence of time. The analysis of such temporal data can be clearly eased by metabolomic approach (Geurts et al., 2017; Roullier et al., 2016). Using such strategy, should drastically improve induction observation and avoid (or decrease) irreproducibility of microbial co-culture experiment, as demonstrated in the *B. obtusa* and *E. lata* co-culture (Azzollini et al., 2018; Glauser et al., 2009).

4.4.Are compound isolation and identification a challenging task?

Metabolomic approaches, as described previously, also help to evaluate the chemical diversity of the compounds induced during microbial interactions. High mass and spectral accuracy information provided by high-resolution mass spectrometry used either in LC-MS or imaging-MS strategies, allow the dereplication (also called annotation) of compounds of interest (Wolfender et al., 2019). The identification of the highlighted metabolites based only on the MS data represents the most challenging step (Bertrand et al., 2014b) because it is a very time consuming work and, as reported, co-culture-induced metabolites display a lower database hit rate in comparison to constitutively produced compounds (Bertrand et al., 2013b). Therefore, such strategies can highlight reported and unreported compounds revealing chemical novelty within induced compounds. As a complementary approach to traditional dereplication strategy, MS/MS spectral similarity networking (Allegra T. et al., 2019; Wang et al., 2016) (also called Molecular Network) represents a very promising approach to reveal the structural relationship between constitutive and induced compounds. Thus, it can highlight structurally analogous metabolites based on similar fragmentation patterns yielding sometimes the identification of a large number of analogous compounds (Oppong-Danquah et al., 2018; Traxler et al., 2013).

Once unreported compound is highlighted, a real challenge consists in isolating it for further identification. Some studies reported the isolation of major induced compound from a very limited amount of raw material (Bertrand et al., 2013c; Bohni et al., 2016), however, to purify and identify minor induced compound a large amount of extract remains necessary. Obtaining such quantity could become a great challenge, especially if reproducibility of microbial co-culture is limited. Bertrand and collaborators reported difficulties observed in upscaling from 2-cm- to 15-cm-diameters Petri dishes the co-culture of *Fusarium* sp. strain with *A. clavatus* (Bertrand et al., 2014a). In their example, some highlighted induced compounds disappeared after up-scaling, particularly early induced ones.

Instead of multiplying the number of replicates to get more chances to obtain appropriate starting material, in some studies, pure-strain culture conditions were evaluated focusing on finding easier solution to access to co-culture induced compounds. Some successful examples exist, with the addition of epigenetic modifiers (Akone et al., 2016) or one of the organisms extract or sterilized organisms (Akone et al., 2016; Ancheeva et al., 2017; El-Sayed et al., 2019; Liang et al., 2019; Rateb et al., 2013). Strategies modifying one of the microorganisms of the co-culture setup may also provide improved production and should be eventually tested (Boruta et al., 2019). Yet, not enough cases are reported to provide a general strategy to access such compounds of interest, and research in this direction should be continued.

Even if many successful examples of compound isolation were reported in the literature, the compound isolation and identification remains a challenging task, in particular in the case of induction of minor compounds.

5. Conclusion

This review provides a comprehensive analysis of NPs induced in microbial co-culture with a specific focus on their chemical structure. As general trends, the induced structures were clustered into 9 significant clusters which can be organized in three groups corresponding to “Non-nitrogenated compounds” (clusters 1-2), “non-alkaloidic nitrogenated compounds” (cluster 3-6), “alkaloidic compounds” (clusters 7-9). This shows that very large variety of structures are induced; even though rather focused physico-chemical properties were observed. Therefore, co-culture can be a promising strategy for the specific production of some valuable NPs and induction of new ones. Among all those compounds, it is interesting to note that many of them exhibit antimicrobial activity, which can indicate that they have a specific role in interspecies microbial interaction. This is further pointed out by the induction of small molecule related to microbial communication, such as butyrolactones (Ancheeva et al., 2017; Chen et al., 2015; Hoshino et al., 2015b; Tata et al., 2015) or oxylipins (Combès et al., 2012). However, more importantly, it is interesting to note that some key chemical scaffolds in regards to interspecies chemical mediation are not reported as induced in co-culture yet. No small terpenes, highly lipophilic compounds related to plant communication (Ta and Arnason, 2015) was reported as induced. The quinolones which act in bacterial quorum sensing (Dubern and Diggle, 2008) were also not observed as induced. Similarly, among reported molecules, only one glucosides, stromemycin (**84**) (Nonaka et al., 2011), was reported as induced. This observation may raise questions about the mechanisms of induction. In case of pathways activation, various initiation mechanisms may be envisaged, such as a response to a chemical signal. In that case, the chemical signal may not be induced itself. Various cases were tentatively explored that may provide some understanding in the induction mechanisms. For example, the induction may be

reproduced by sterilizing one of the partner (Ancheeva et al., 2017; Liang et al., 2019; Rateb et al., 2013), but sometime specific interaction with living microorganisms is necessary (Angell et al., 2006). Unfortunately, only rare examples exist where the authors explore how specific is the interaction. Such studies should be conducted in depth in the future to provide new insight in the induction mechanisms.

Yet, based on the reported co-culture induced metabolites, as compared with other strategies traditionally used in microbial NPs research (i.e. OSMAC approach), no prediction can be done on what kind of scaffold can be induced. One exception is the production of “linear polyketides, Oxylipins and Fatty acids” and “Cyclic peptides, diketopiperazines and related compounds” that seems to occurs mostly during liquid fermentation independently of the type of organisms. In fact, this is highly dependent on both the biosynthetic capabilities of both the co-cultured organisms, and also to the couple of micro-organisms used. Thus, to the best of our knowledge, all culture-based induction strategies are unfortunately still based on serendipity. In addition, the literature survey achieved here also highlights the lack of information provided by articles studying co-culture. As a general guideline for future co-culture articles, additional information should be provided such as:

- a clear comparison with mono-culture chemical profiles,
- the number of replicates used during the study,
- the induction levels of all reported compounds (fold change of up-regulation or *de novo* induction)
- the presence of other similar compounds produced by the mono-cultures.

Such information, should help delineating the mechanism of induction for comparison with other studies.

Most chemical novelty is still discovered from exploring novel microbial sources, even if all compounds are not produced under standard laboratory conditions. In addition, chemical novelty is also easily obtained by OSMAC approach, albeit it is not an exhaustive strategy. While monitoring of induction is nowadays achievable, no clear study comparing chemical novelty access or coverage from microbial co-culture or OSMAC is available. Therefore, comparing those strategies remains difficult to achieve, and this provides new directions that need to be explored in the near future. Furthermore, combining both strategies was also not yet applied to extend explore microbial chemical space. This may, in addition to highlight novel compounds, provide information about NPs production regulation mechanisms.

In addition, in comparison to the screening of novel microbial sources or OSMAC approach applied to one microbial strain, co-culture still suffers from difficulties for large scale production and structural identification of those compounds. The single strain culture approaches present the great advantage that up-scaling for further purification and structural elucidation is rather easy to achieve. In the case of microbial co-culture, the up-scaling is still rather complicated and all induced compounds may not be observed after up-scaling, in particular for minor induced compounds (Bertrand et al., 2014a). This may also be due to difficulties in obtaining stable large scale microbial consortium (Stephens et al., 2019). Thus, up-scaling strategies need to be explored in-depth to provide access to all induced compounds in large scale. Unfortunately, this step remains highly time-consuming. Such up-scaling problems are sometimes related to the ignorance of the organisms responsible for co-culture induced compound production. Therefore, exploring biosynthetic gene cluster activation under co-culture conditions (Schroeckh et al., 2009; Torres-Monroy and Ullrich, 2018) is very crucial. Thus, transcriptomic studies should be used as often as possible to be able to link compound induction

with gene transcription. Such an approach may provide novel strategies for specific compound production such as heterologous expression (Gomez-Escribano and Bibb, 2014), homologous activation (Zhang, B. et al., 2017).

In conclusion, to make use of microbial co-culture at the same level as the OSMAC approach, more studies still need to be engaged to provide solutions to overcome the previously discussed bottlenecks. Overcoming those aspects of the co-culture strategy will definitely help future drug discovery programs, as well as studies of microbial community in a chemical ecology viewpoint.

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Figure Legends

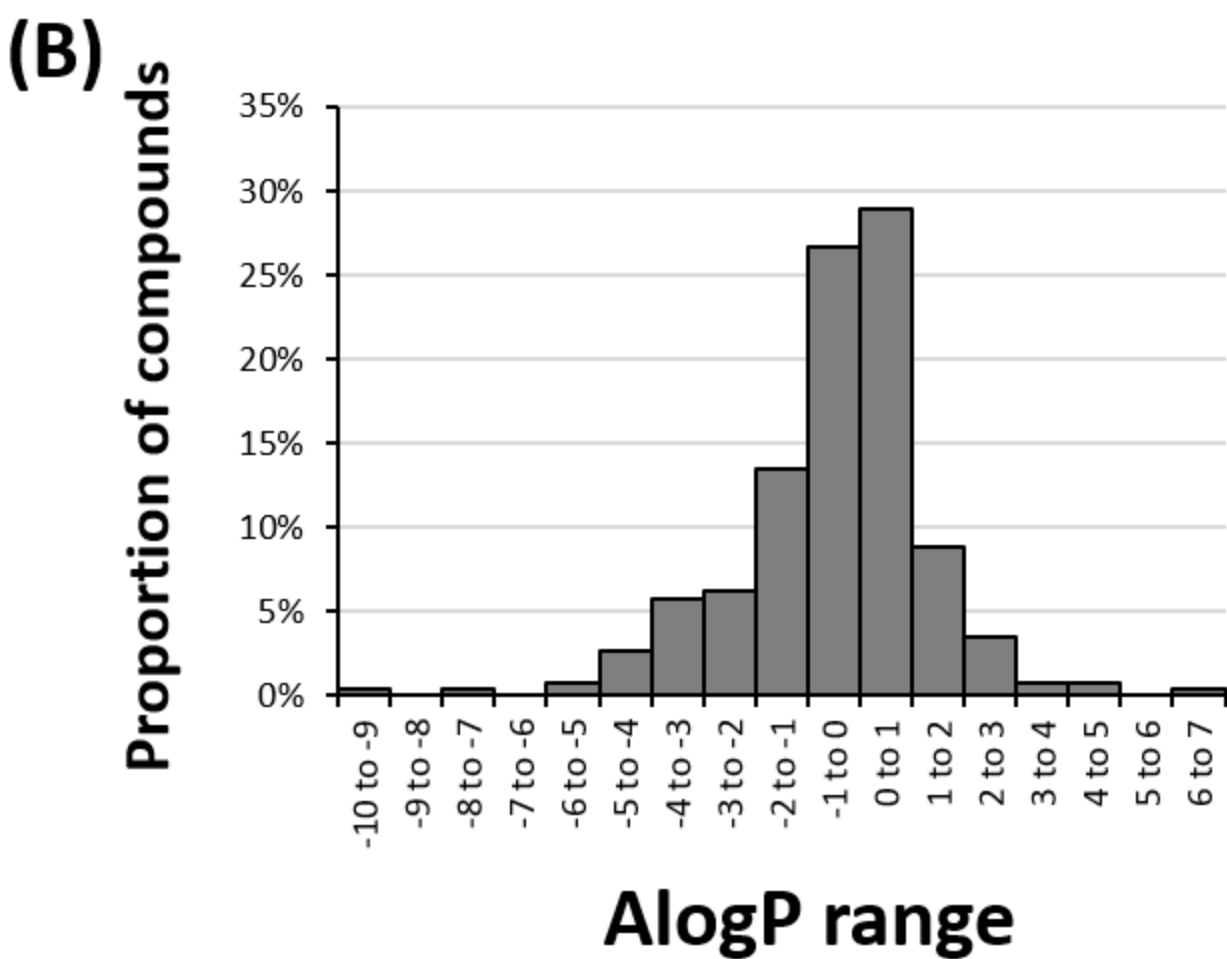
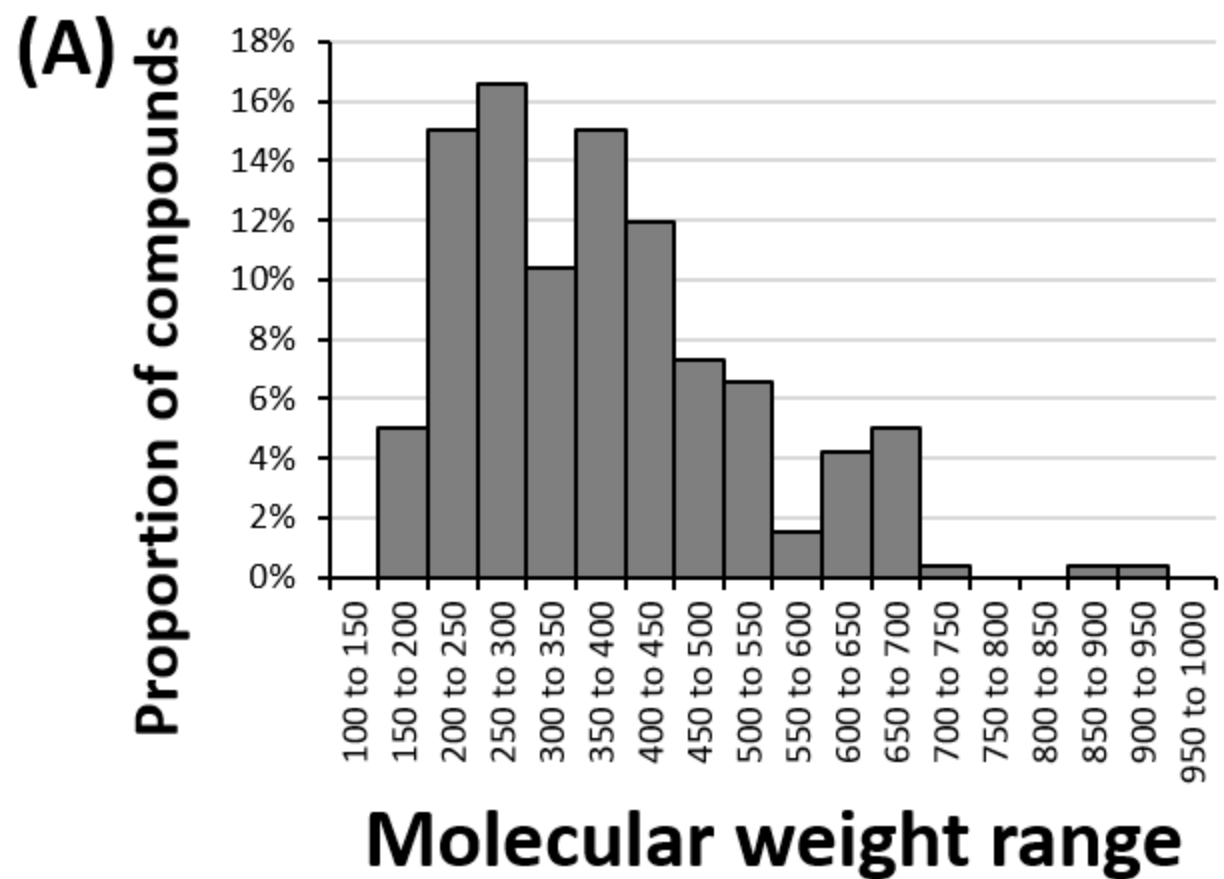
Figure 1. Distribution of molecular weight (**A**) and lipophilicity (AlogP) (**B**) calculated using Cytoscape (Shannon et al., 2003) and the ChemViz2 plugin (<http://www.rbvi.ucsf.edu/cytoscape/chemViz2/>).

Figure 2. Structural similarity network of structures reported to be induced during microbial co-culture based on Tanimoto Index. Solid line edges correspond to Tanimoto Index higher or equal to 0.7 (the line width indicates how similar the structures are – larger width indicates more structural similarity); dash line edges indicated a forced link in the case of lonely structures. The network is available in Cytoscape file format as supplementary information for in depth exploration (<https://gitlab.univ-nantes.fr/bertrand-s-1/structural-network-of-co-culture-induced-compounds>).

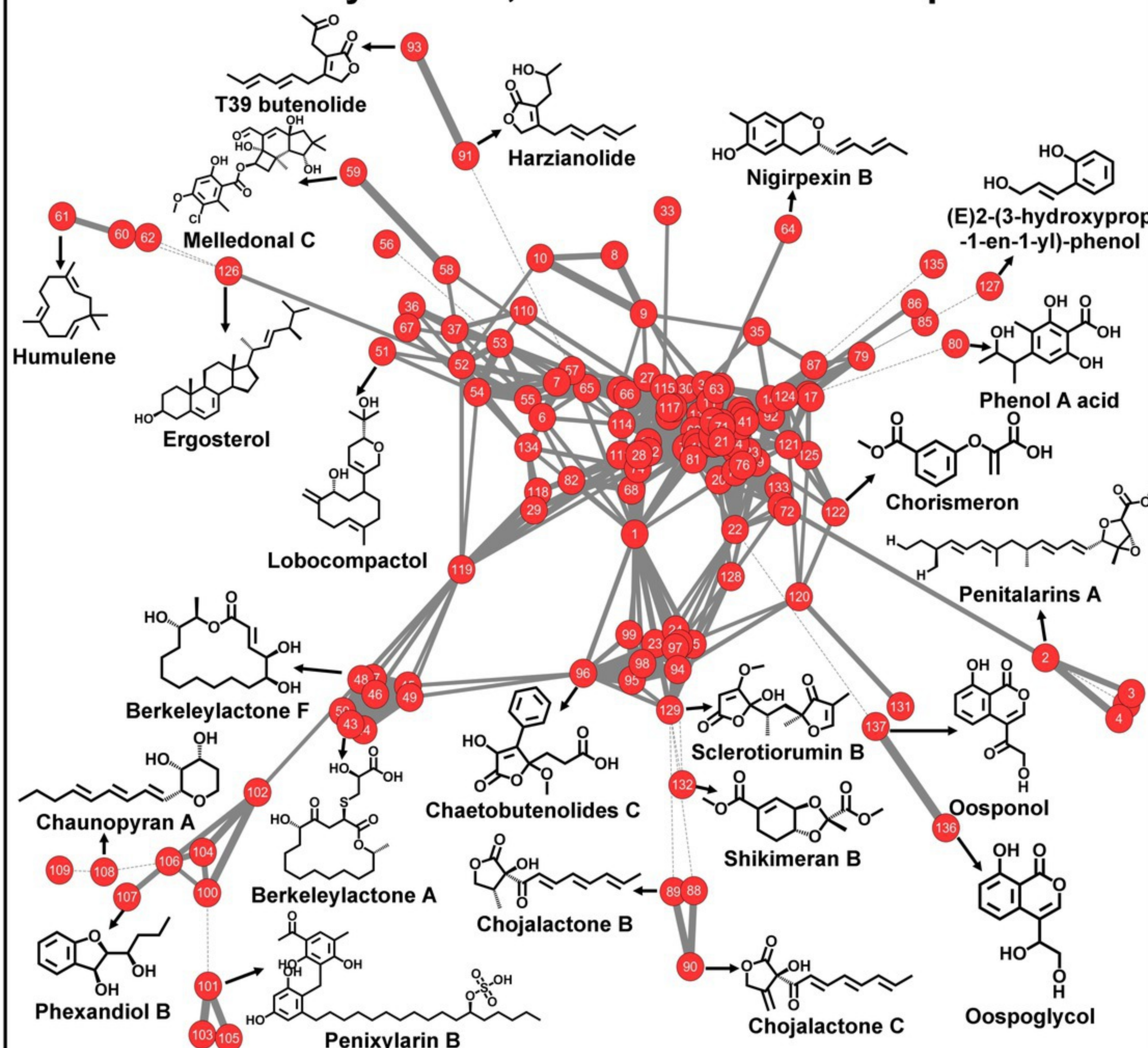
Figure 3. Structural similarity network of Cluster 1 using a Tanimoto Index higher or equal to 0.8 (the line width indicates how similar the structures are – larger width indicates more structural similarity). The network is available in Cytoscape file format as supplementary information for in depth exploration.

Figure 4. Distribution of co-culture microbial couples used in reported experiments.

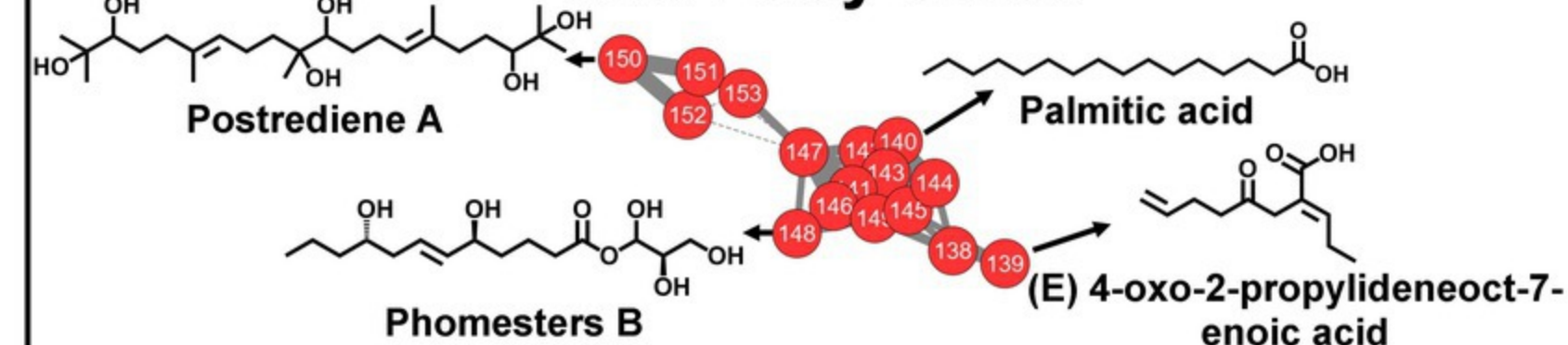
Figure 5. Distribution of relevant biological activity (< 10 μ M) reported for all co-culture induced compounds (complementary information about distribution taking into consideration moderate activity are presented in Figure S3).



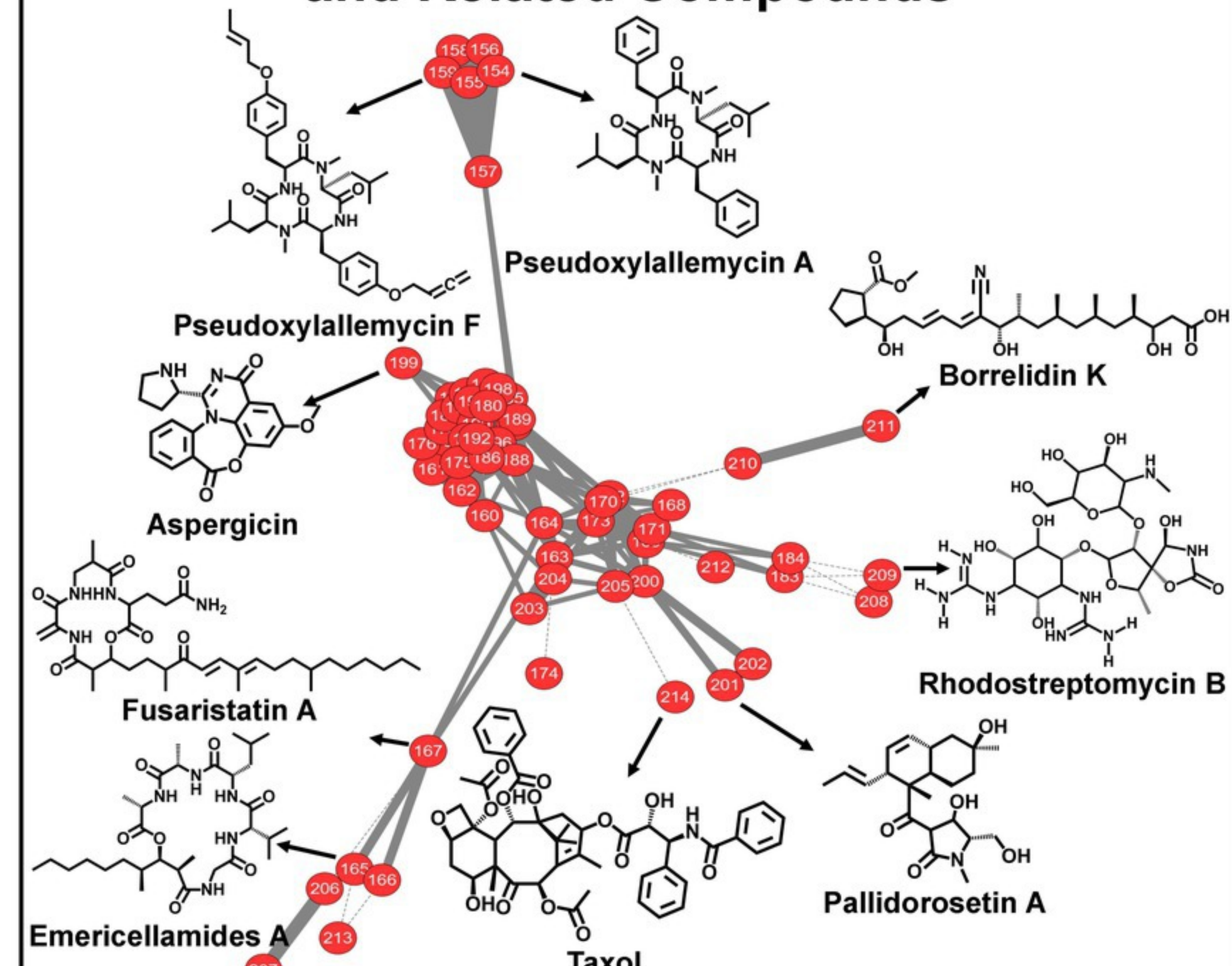
Cluster 1: Polyketides, Macrolides and Diterpenoids



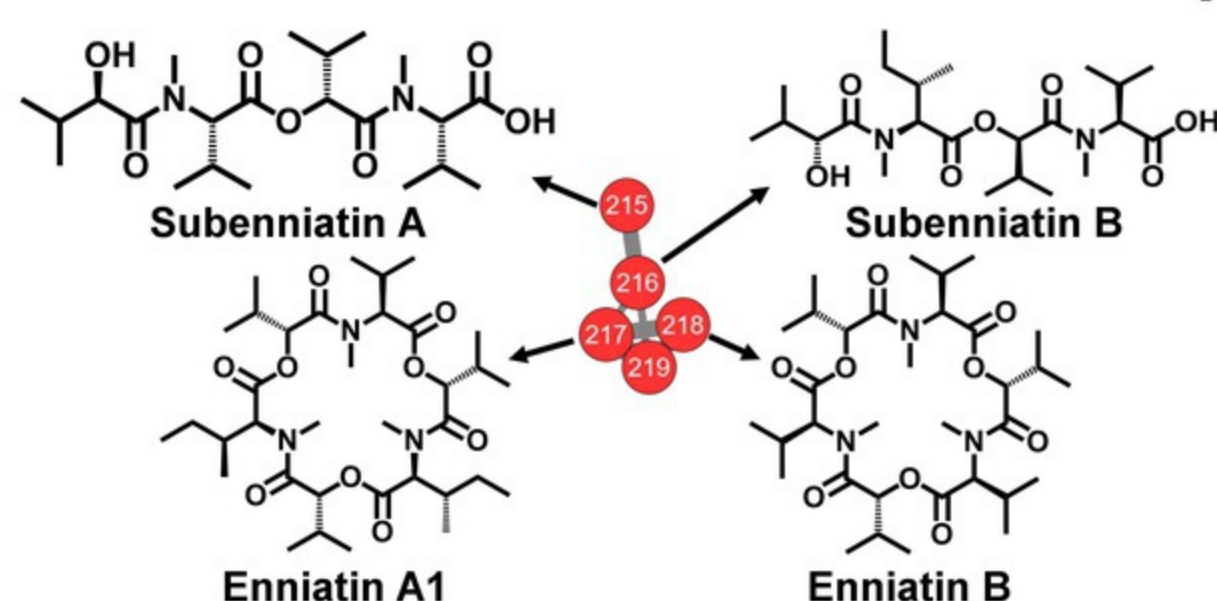
Cluster 2: Linear Polyketides, Oxylipins and Fatty acids



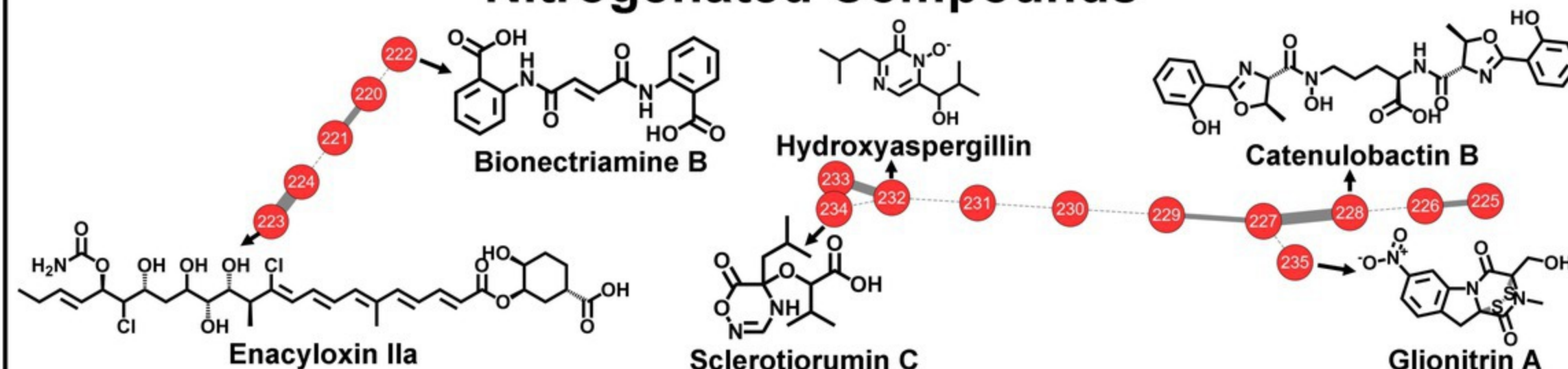
Cluster 3: Cyclic Peptides, Diketopiperazines and Related Compounds



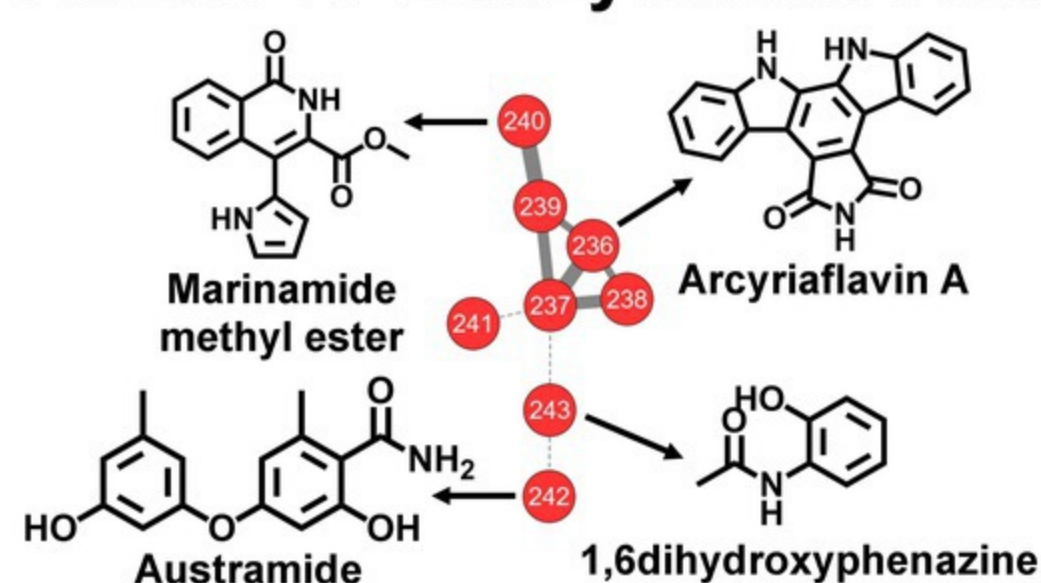
Cluster 4: Other Non-Ribosomal Peptides



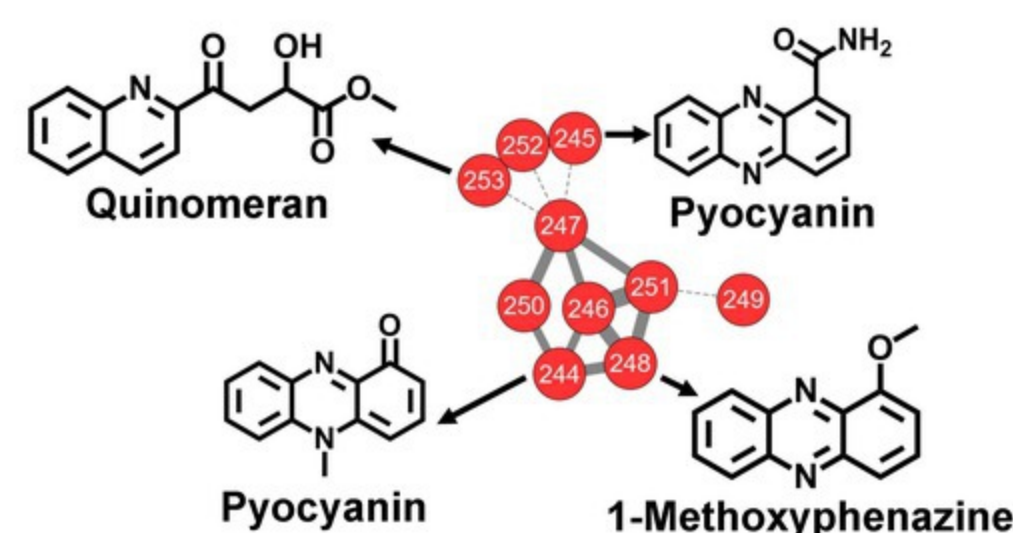
Cluster 5 and 6: Miscellaneous Non-Alkaloidic Nitrogenated Compounds



Cluster 7: Benzylamine Alkaloids



Cluster 8: Phenazine Alkaloids



Cluster 9: Tryptamine and Pyrrole Alkaloids

