A versatile yeast model identifies the pesticides cymoxanil and metalaxyl as risk factors for synucleinopathies

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HIGHLIGHTS

- Screening of pesticides identified potential risk factors for the development of synucleinopathies.
- In a yeast cell model, cymoxanil and metalaxyl caused aSyn mislocalization, leading to aggregation in the cytoplasm.
- Cymoxanil and metalaxyl promoted the formation of aSyn inclusions in a mammalian cell aggregation model of synucleinopathies.
- Both cymoxanil and metalaxyl were found to decrease cell viability and to increase the number of apoptotic cells.

GRAPHICAL ABSTRACT
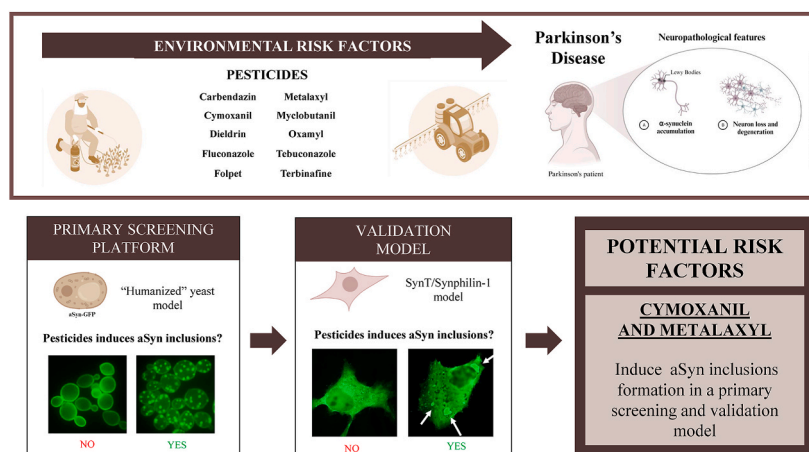
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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons and the presence of Lewy bodies, which predominantly consist of aggregated forms of the protein alpha-synuclein (aSyn). While these aggregates are a pathological hallmark of PD, the etiology of most cases remains elusive. Although environmental risk factors have been identified, such as the pesticides dieldrin and MTPT, many others remain to be assessed and their molecular impacts are underexplored. This study aimed to identify pesticides that

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could enhance aSyn aggregation using a humanized yeast model expressing aSyn fused to GFP as a primary screening platform, which we validated using dieldrin. We found that the pesticides cymoxanil and metalaxyl induce aggregation of aSyn in yeast, which we confirmed also occurs in a model of aSyn inclusion formation using human H4 cells. In conclusion, our approach generated invaluable molecular data on the effect of pesticides, therefore providing insights into mechanisms associated with the onset and progression of PD and other synucleinopathies.

1. Introduction

Pesticides are a diverse group of chemicals that are widely used for pest control, including insecticides, herbicides, fungicides, and rodenticides (Bonner and Alavanja, 2017). While they have played a crucial role in increasing food production and combating vector-borne diseases, their indiscriminate and intensive use has led to environmental contamination and adverse health effects (Carvalho, 2017; Nicolopoulou-Stamati et al., 2016). Concerningly, the global usage of pesticides is projected to steadily increase by 0.5% every year, reaching 4.4 million tons by 2026. Despite mandatory risk assessments and safety evaluation, lack of coherence between countries has resulted in contradictory reports, and questionable "safe limits" (Tweedale, 2017). Although occupational activity is the main source of pesticide exposure, their widespread use in agriculture, homes, and public gardens puts the entire population at risk. In fact, the European Food Safety Authority (EFSA) has reported that many food samples consistently contain pesticide residues above legal limits of maximum residue levels (MRLs) (Richardson et al., 2019). Pesticides have also been detected in human breast milk, which raises concerns about their impact on post-natal development (Lu et al., 2015). Alarmingly, chronic exposure to even low pesticide doses can lead to various health complications, such as gastrointestinal, carcinogenic, respiratory, reproductive, endocrine, and neurological disorders (Mostafalou et al., 2013). Indeed, environmental contaminants such as pesticides as well as metals, and solvents have been associated with the development of neurodegenerative diseases (Cannon and Greenamyre, 2011; Chin-Chan et al., 2015; Kwok, 2010; Shvachiy et al., 2023).

Several epidemiological studies associated exposure to dieldrin, paraquat, rotenone, maneb, and ziram with increased risk of Parkinson's disease (PD) (Cao et al., 2018; Freire and Koifman, 2012), part of a heterogeneous group of disorders referred to as synucleinopathies (Kim et al., 2014). In PD, there is a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and a decline of dopamine (DA) levels in the striatal neurons. The onset and progression of PD involve several cellular mechanisms, including oxidative stress, excitotoxicity, impaired proteasomal and lysosomal pathways, and the formation of intraneuronal eosinophilic inclusions known as Lewy Bodies (LB) containing insoluble protein aggregates of phosphorylated tau (p-tau) and amyloid beta protein (Aβ) (Ross and Poirier, 2004; Soto, 2003), but of mainly alpha-synuclein (aSyn) (Walker et al., 2019; Breydo et al., 2012).

aSyn, encoded by the *SNCA* gene, is a small natively unfolded protein expressed in presynaptic neuron terminals. Although its physiological function is not fully understood, it has been established that it plays a role in synaptic trafficking and vesicle budding, as well as SNARE complex formation and DA release (Kim et al., 2014; Longhena et al., 2017). Under normal physiological conditions, aSyn exists in a dynamic equilibrium of monomers and helical oligomers (tetramers) but, in disease, it aggregates into insoluble fibrils (Longhena et al., 2017). The exact triggers for this abnormal aggregation remain unclear, but mutations, abnormal expression levels, truncation, dysfunction of clearance pathways, and post-translational modifications are considered plausible explanations. Another factor that can promote aSyn oligomerization is exposure to pesticides, normally through oxidative stress, ubiquitin-proteasome inhibition, mitochondria dysfunction, ROS accumulation, and altered lipid metabolism (Antony et al., 2013). Rapidly identifying environmental factors contributing to PD development is therefore critical, given the current increasing exposure to such hazardous contaminants.

Traditionally, neurotoxicity assessment is based on animal experimentation, but this approach is expensive, time-consuming, and raises ethical concerns. Yeast cells, on the other hand, are easy to use, genetically tractable, and widely exploited in high-throughput studies and as a primary drug-screening platform (Tenreiro and Outeiro, 2010; Tenreiro et al., 2017; Delenclos et al., 2019; Su et al., 2010). Of note, the development of a "humanized yeast" model for synucleinopathies has facilitated our understanding of the disease and the cellular processes affected by aSyn. In yeast, aSyn overexpression results in dose-dependent cytotoxicity: when 1 copy is expressed, aSyn is associated with the plasma membrane with no cytotoxic effect; when 2 or more copies are expressed, aSyn localization shifts to the cytoplasm, where it aggregates into vesicles analogous to LB, becoming cytotoxic (Outeiro and Lindquist, 2003). The "*S. cerevisiae* toolbox" has already provided insights into understanding PD pathogenesis by identifying ER-to-Golgi trafficking defects, impairment of the quality control systems, as well as alterations in lipid metabolism, mitochondrial stress, chronological life span, and induction of autophagy and mitophagy as associated with aSyn aggregation and cytotoxicity (Outeiro and Lindquist, 2003; Gitler et al., 2008; Cooper et al., 2006; Sampaio-Marques et al., 2012). Yeast expressing human aSyn has also been used in screens to identify genes or chemical compounds that can reduce aSyn-induced cytotoxicity, with the goal of uncovering novel treatments (Su et al., 2010; Tardiff and Lindquist, 2013; Tardiff et al., 2014). Applying the reverse principle, our research aims to identify pesticides that may potentially contribute to the development and progression of PD. Our results unveiled that cymoxanil and metalaxyl induced aSyn mislocalization, leading to its aggregation in the cytoplasm, which was further confirmed through a mammalian cell aggregation model. Thus, we identified cymoxanil and metalaxyl as environmental risk factors for the onset and progression of PD.

2. Materials and methods

2.1. Yeast strains

The *Saccharomyces cerevisiae* strains used in this study, along with their respective genotypes, are detailed in Table 1.

To generate a strain harboring only 1 copy of the *SNCA* gene, a strain with 2 genomic copies of the fusion *GAL1pr-SNCA(WT)-GFP* was mated

Table 1
List of *S. cerevisiae* strains used in this work.

Strain	*SNCA* gene	Genotype	Source
W303	0 copies	*MATα (can1-100 his3-11 15 leu2-3112 trp1-1 ura3-1 ade2-1)*	Euroscarf
W303 SNCA (WT)-GFP	2 copies	*MATa (ura3-1::GAL1pr-SNCA(WT)-GFP URA3+ trp1-1::GAL1pr-SNCA (WT)-GFP TRP1+)*	Sancenon et al. (2012)
W303 SNCA (WT)-GFP	1 copy	*MATa (ura3-1::GAL1pr-SNCA(WT)-GFP URA3+)*	This study

with a wild-type strain of the opposite mating type. After sporulation and tetrad dissection, a strain encoding only 1 copy of the *SNCA* gene was isolated and selected based on genotyping results.

2.2. Growth conditions

Cells were grown overnight in an orbital shaker, at 30 °C, 200 rpm, in Synthetic Complete (SC) medium [5.0 g/L ammonium sulfate $((NH_4)_2SO_4)$, 1.7 g/L yeast nitrogen base w/o amino acids and w/o ammonium sulfate $((NH_4)_2SO_4)$, 1.4 g/L dropout mix lacking leucine, histidine, tryptophan, and uracil; 0.4 g/L leucine; 0.08 g/L histidine; 0.08 g/L tryptophan; 0.08 g/L uracil] with 20 g/L of raffinose in a ratio of flask volume/medium of 5:1. Cells were diluted in SC medium with 10 g/L of galactose for promoter induction, or 20 g/L of glucose for promoter repression. Where indicated, cells were exposed to the pesticides (Sigma-Aldrich) described in Table S1. Pesticides were stored in light-protected aliquots and resuspended in dimethyl sulfoxide (DMSO) immediately prior to usage. The DMSO concentration never exceeded 0.5% (v/v).

2.3. Microbroth dilution assay

A microbroth dilution assay was used to determine the minimum inhibitory concentration of pesticides. Yeast cells from an overnight culture grown in SC-raffinose (OD_{600nm} between 1 and 2) were diluted to an OD_{600nm} of 0.05 in SC medium containing 10 g/L of galactose and various pesticide concentrations. After incubation at 30 °C for 24 h, OD_{600nm} was measured using a microplate reader (Spectra max Plus Molecular Devices).

2.4. Fluorescence microscopy

aSyn localization and aggregation was evaluated by fluorescence microscopy (Leica Microsystems DM-5000B) with a 100× oil immersion objective. Images were obtained with a Leica DFC350 FX Digital Camera and processed with LAS X Microsystems software.

2.5. Cell line culture and transfection

Human neuroglioma cells (H4) were cultured in P75 flasks with Opti-MEM I reduced serum medium (Life Technologies-Gibco) supplemented with 10% fetal bovine serum (FBS; PAN-Biotech) and 1% penicillin–streptomycin (PS; PAN-Biotech). Cultures were maintained in a 37 °C incubator with 5% CO_2 and controlled humidity. For transfection, approximately 80,000 cells were plated per well in a 12-well plate (Costar, Corning) and allowed to grow for 24 h, after which the medium was refreshed. Cell transfection was performed using Fugene, according to the manufacturer's recommendations (Promega). Briefly, 100 μL of Opti-MEM I medium without FBS and PS was mixed with 3 μL of Fugene and incubated for 5 min at room temperature (RT). Subsequently, 1 μg of SynT and synphilin-1 DNA, at a ratio of 1:3, was added to the mixture and incubated for 30 min at RT. A total of 100 μL of each mixture was carefully dispensed dropwise into each well, and cells were further incubated for 48 h. When required, cymoxanil and metalaxyl, dissolved in DMSO immediately prior to use, were added 24 h post-transfection, in final concentrations of 1, 10 or 20 μM. The DMSO concentration in all cases was always lower than 0.1% (v/v).

2.6. Immunocytochemistry

Forty-eight hours after transfection, cells were washed with 1× phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 30 min at RT. After fixation, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 20 min at RT. Subsequently, a blocking step with 3% bovine serum albumin (Nzytech) in 1 × PBS for 1 h was performed. Then, cells were incubated overnight at 4 °C with

primary antibody mouse anti-ASYN (1:1000, BD Transduction Laboratory), previously shown to be specific for ASYN (Masaracchia et al., 2020; Dominguez-Meijide et al., 2021; Lázaro et al., 2014), which we confirmed by Western Blot (not shown). The following day, cells were washed and incubated with the Alexa Fluor 488 goat anti-mouse secondary antibody (Life Technologies-Invitrogen) for 2 h at RT. Finally, to visualize cellular nuclei, the cells were stained with DAPI (Carl Roth) at a dilution of 1:5000 in 1× PBS for 10 min. Subsequently, coverslips were mounted onto SuperFrostR microscope slides, treated with Mowiol (Calbiochem) and allowed to dry. The slides were then stored at RT until further imaging and analysis.

2.7. Image acquisition and analysis

Images were acquired utilizing an epifluorescence microscope Zeiss Axio Observer equipped with a 100× oil objective lens. A total of 30 images were acquired for each experimental condition, employing predefined settings tailored to each staining protocol. For quantification of the number and area of the aSyn inclusions, a "binary" black and white image is required, where a threshold can be applied, and the inclusions are differentiated from the background. Subsequent image analysis was performed using the "Analyse particles" plugin in the Fiji open-source software.

2.8. Quantification of aSyn inclusions

Transfected cells were assessed by examining the pattern of aSyn inclusions and categorized into four distinct groups: cells devoid of inclusions, those with 1–4 inclusions, those harboring 5 to 9 inclusions, and those displaying 10 or more inclusions. Results were expressed as the percentage relative to the total count of transfected cells, obtained from three independent experiments.

2.9. Quantification of apoptotic cells

Apoptotic cells were assessed by monitoring alterations in nuclear morphology following DAPI staining, with a total of 300 cells counted per condition. Results were expressed as the percentage of cells exhibiting characteristic nuclear morphology changes associated with apoptosis. The results were obtained from three independent experiments.

2.10. Cellular viability

Cellular viability was evaluated using the trypan blue assay. Briefly, cells from each condition were collected, mixed with 0.4% of trypan blue dye (1:1 ratio) and quantified through an automated cell counter (Countess from Invitrogen). Results were subsequently standardized to the control and expressed as the percentage of viable cells, as determined from three independent experiments.

2.11. Statistical analyses

Data were analysed using GraphPad Prism 5 (San Diego California, USA) software and expressed as the mean ± SD of at least three independent experiments.

3. Results and discussion

3.1. Screening of pesticides as potential risk factors for synucleinopathies

To identify pesticides that could potentially contribute to the onset and progression of PD, we exposed a strain of *S. cerevisiae* harboring one copy of the aSyn-GFP fusion gene to increasing concentrations of different classes of pesticides. These encompassed organochlorine, acylalanine, azole, urea, benzimidazole, allylamine, carbamate and
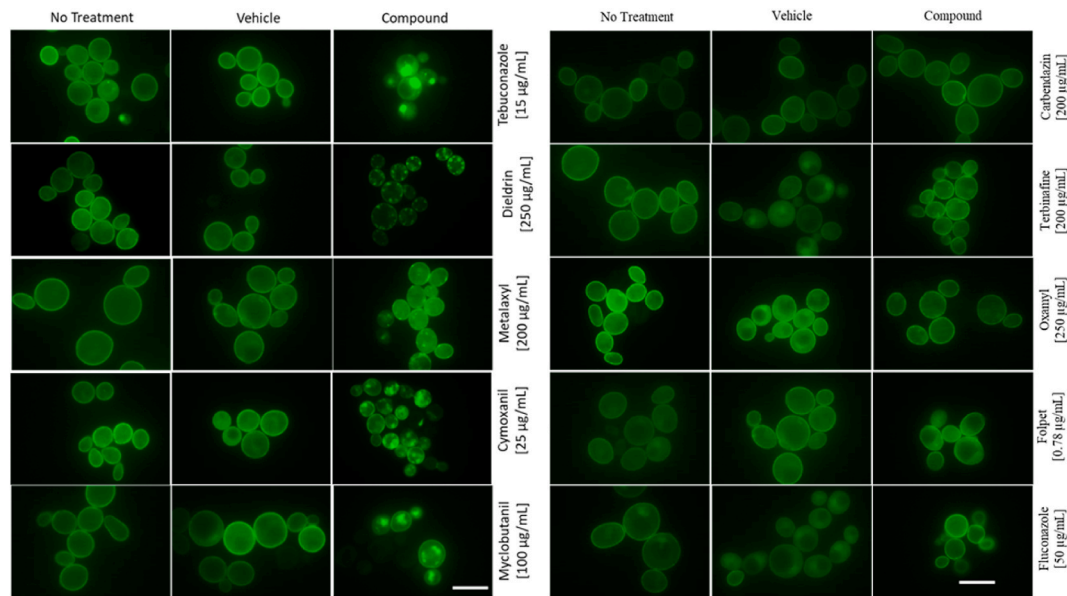
Fig. 1. The effect of pesticides on aSyn localization and formation of inclusions. *S. cerevisiae* cells harboring one copy of aSyn-GFP were grown in SC medium with 20 g/L of raffinose at 30 °C 200 rpm, diluted to an OD_{600nm} of 0.05 in SC medium with 10 g/L of galactose in 96-well round bottom plates, and immediately exposed to increasing concentrations of pesticides (diluted in DMSO), as well as a pesticide-free control. After incubation at 30 °C for 24 h, aSyn localization and inclusion formation were analysed by fluorescence microscopy (Bar, 5 μm).

Table 2
Summary of the pesticides tested and respective phenotypes. The highest tested concentration, the MIC, the lowest concentration inducing aSyn aggregation, and the resulting phenotype of *S. cerevisiae* harboring one copy of the aSyn-GFP are provided. A lack of growth inhibition or aSyn aggregation at the tested concentrations is denoted by (−).

Pesticide	Maximum concentration tested (μg/mL)	MIC (μg/mL)	Minimum concentration for aSyn aggregation (MAC) (μg/mL)	Phenotype
Tebuconazole	15	−	0.23	**I. aSyn aggregation** without growth inhibition
Dieldrin	250	−	7.8	
Metalaxyl	200	−	200	
Cymoxanil	200	50	6.25	**II. aSyn aggregation** and growth inhibition
Myclobutanil	200	200	6.25	
Carbendazim	200	−	−	**III. No aSyn aggregation** and no growth inhibition
Terbinafine	200	−	−	
Oxamyl	250	−	−	
Folpet	50	0.78	−	**IV. No aSyn aggregation** and growth inhibition
Fluconazole	200	50	−	

phthalimide compounds (Table S1).

We assessed the cytotoxicity of pesticides by microbroth dilution to establish the minimum inhibitory concentration (MIC), the lowest concentration that completely prevented visible growth of the strain. Concurrently, we analysed aSyn localization and the formation of inclusions by fluorescence microscopy. To be considered a positive hit, the pesticide should induce a shift in aSyn localization from the plasma membrane to the cytoplasm and stimulate aSyn inclusion formation. The tested pesticides exhibited diverse impacts on cell growth and aSyn localization and inclusion formation, resulting in distinct phenotypes. These outcomes were categorized into four groups (Fig. 1 and Table 2).

Group I pesticides, which includes dieldrin, metalaxyl and tebuconazole, led to aSyn inclusion formation without inhibiting cell growth at the tested concentrations. (Fig. 1 and Table 2). In group II, cymoxanil and myclobutanil led to both cell growth inhibition and aSyn inclusion formation, whereas carbendazim, terbinafine and oxamyl, classified as group III, did not lead to either. Finally, folpet and fluconazole, in group IV, inhibited cell growth but did not promote aSyn aggregation, demonstrating that aggregation is not an unspecific effect of cellular stress (Fig. 1 and Table 2). In summary, we found that cymoxanil, metalaxyl, tebuconazole, myclobutanil and dieldrin increased aSyn aggregation. Dieldrin led to a distinct pattern, with smaller aggregates and mostly around the cell periphery even after exposure to the highest concentration, while the other pesticides mostly led to larger inclusions in the cytosol. We also observed that aggregation was not associated with toxicity in all scenarios. This could be due to relatively low aSyn levels (cells express only 1 copy of aSyn-GFP) or reflect a different nature of aggregates (Alam et al., 2019; Stefanis, 2012; Perrino et al., 2019; Raiss et al., 2016).

Tebuconazole and myclobutanil are known to affect the ergosterol biosynthesis pathway, which can alter plasma membrane composition (Lamb et al., 1999). Indeed, given the mode of action of azole compounds (Kwok and Loeffler, 1993) and the fact that aSyn is a lipid-bound protein (Outeiro and Lindquist, 2003), it would be challenging to ascertain whether the observed phenotype resulted directly from an effect on aSyn or was a secondary outcome resulting from plasma membrane alterations, particularly in the case of tebuconazole, which is a stronger inhibitor, and thus these pesticides were excluded from follow up studies.

In all, we concluded that cymoxanil and metalaxyl may constitute possible previously uncharacterized environmental risk factors for synucleinopathies and confirmed a predicted effect of dieldrin in increasing PD risk through aSyn aggregation.

3.2. Cymoxanil and dieldrin induce dose-dependent formation of aSyn inclusions in a yeast synucleinopathy model

Cymoxanil is an acetamide fungicide introduced in the 70s, commonly used to combat downy mildew and late blight diseases in various crops, either alone or in combination with other fungicides (Genet and Vincent, 1999). While its precise biochemical mode of action remains poorly understood, it is established that cymoxanil indirectly affects nucleic acids and amino acid synthesis (Leroux, 1999) and
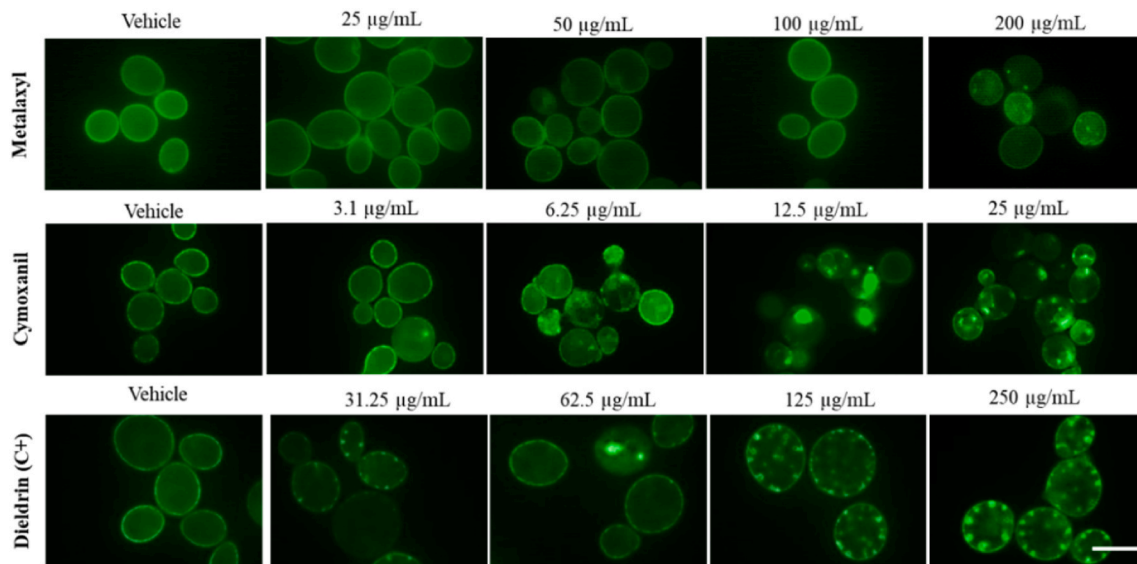
Fig. 2. aSyn localization and inclusion formation upon treatment with cymoxanil, metalaxyl and dieldrin. *S. cerevisiae* cells harboring one copy of aSyn-GFP were grown in SC medium with 20 g/L of raffinose at 30 °C 200 rpm, diluted to an OD$_{600nm}$ of 0.05 in SC medium with 10 g/L of galactose in 96-well round bottom plates, and immediately exposed to cymoxanil, metalaxyl or dieldrin (diluted in DMSO), as well as a pesticide-free control. After incubation at 30 °C for 24 h, aSyn localization and inclusion formation were analysed by fluorescence microscopy (Bar, 5 μm).

inhibits cellular respiration (Ribeiro et al., 2000). We found that 50 μg/mL of cymoxanil inhibited cellular growth and aSyn inclusion formation was already visible in cells exposed to the lowest concentration of 6.25 μg/mL (Fig. 2, Table 2).

Metalaxyl is a broad-spectrum acylalanine fungicide that blocks the incorporation of uridine into RNA, which consequently inhibits rRNA synthesis (Fisher and Hayes, 1982). Although slightly persistent in the environment, metalaxyl has been found in edible food and water. In fact, 46% of surface water samples were found to contain metalaxyl in concentrations reaching 0.191 μg/L, above the EU Maximum Allowable Concentration which is 0.1 μg/L (Allinson et al., 2015). Although metalaxyl neither inhibited yeast cell growth nor induced dose-dependent aSyn formation in the concentrations tested, it promoted the formation of aSyn inclusions at the highest concentration (200 μg/mL) (Fig. 2, Table 2).

As a control, we used the pesticide dieldrin, extensively linked to PD (Richardson et al., 2006; Vale et al., 2003). This compound is a highly lipophilic and persistent organochlorine insecticide known for its
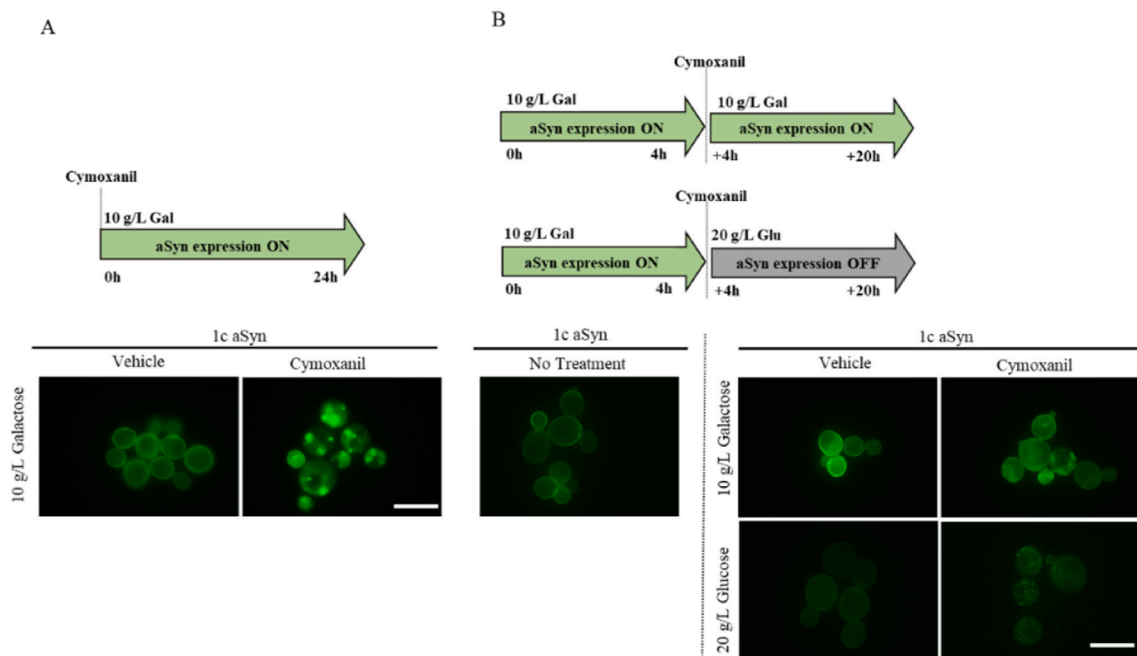

Fig. 3. Cymoxanil leads to intracellular accumulation of aSyn previously localized in the plasma membrane. *S. cerevisiae* cells harboring one copy of aSyn-GFP were grown in SC medium with 20 g/L of raffinose at 30 °C 200 rpm, then diluted to an OD$_{600nm}$ of 0.1 in SC medium with 10 g/L of galactose. A) Cells were exposed immediately to 12.5 μg/mL of cymoxanil for 24 h. B) aSyn expression was induced for 4 h in 10 g/L of galactose medium. Then, cells were maintained in the same medium or transferred to 20 g/L of glucose to repress expression and exposed to 12.5 μg/mL of cymoxanil. In both conditions, cymoxanil was diluted in DMSO, and a pesticide-free control was also added. After incubation at 30 °C, aSyn localization and inclusion formation were analysed by fluorescence microscopy (Bar, 5 μm).
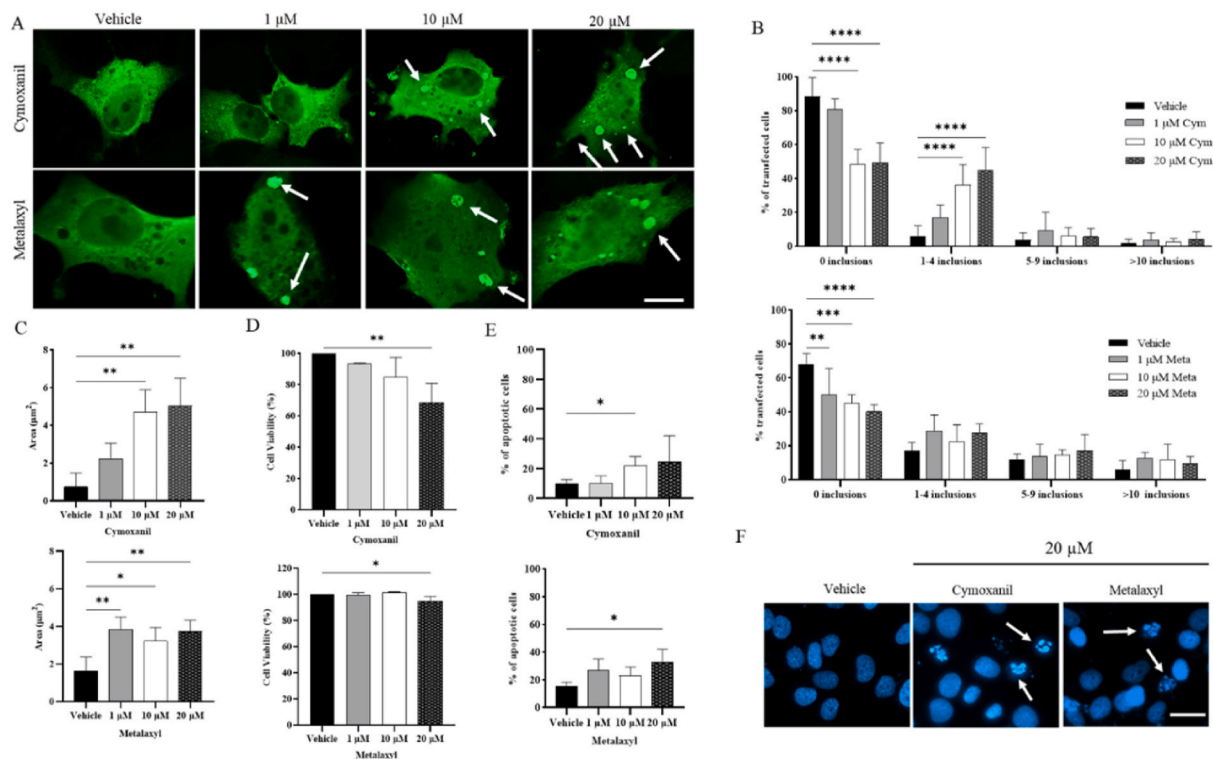
Fig. 4. Cymoxanil and metalaxyl induce formation of aSyn inclusions in a human cell model of synucleinopathies. A) Representative images of cells co-expressing SynT and synphilin-1 treated with cymoxanil and metalaxyl for 24 h (Bar, 5 μm). B) Inclusion quantification. At least 40 cells were scored per experiment and classified into different groups according to the number of aSyn inclusions. The data are presented as mean ± SD of 4 independent experiments. Asterisks (Two-Way ANOVA **p < 0.01, ***p < 0.001 and ****p < 0.0001) depict significant differences relative to the control. C) Average area of the aSyn inclusions in cells positive for aSyn expression. The data are presented as mean ± SD of 3 independent experiments. Asterisks (One-Way ANOVA * p < 0.05 and **p < 0.01) depict significant differences relative to the control. D) Cell viability was assessed by trypan blue assay after 24 h of treatment with cymoxanil and metalaxyl. The data normalized to the control are presented as mean ± SD of 3 independent experiments. Asterisk (One-Way ANOVA **p < 0.01) depict significant differences relative to the control. E) Nuclear morphology was assessed by DAPI staining where 300 cells were counted per experiment after a 24 h treatment with cymoxanil or metalaxyl. The data are presented as mean ± SD of 3 independent experiments. Asterisk (One-Way ANOVA **p < 0.01) depict significant differences relative to the control. F) Representative images of cells displaying nuclear morphology changes associated with apoptosis upon treatment with cymoxanil or metalaxyl for 24 h (Bar, 5 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inhibition of the GABA(A) receptors, resulting in hyperexcitation and a massive Ca^{2+} influx (Vale et al., 2003). Despite its ban in the USA in the late-80s due to its adverse effects and persistence in the environment (up to approximately 25 years) (Meijer et al., 2001), dieldrin continues to be used in non-developed countries. Its continued presence in the environment presents health risks to the general population due to its bio-accumulative nature and persistence (Jorgenson, 2001). Although dieldrin did not affect cellular growth at any of the tested concentrations, it did induce aSyn inclusions at concentrations exceeding 7.8 μg/mL (Fig. 2 and Table 2).

3.3. Cymoxanil leads to intracellular accumulation of aSyn previously localized in the plasma membrane

In yeast cells, human wild type aSyn typically undergoes intracellular trafficking, and is delivered to the plasma membrane via the secretory pathway (Dixon et al., 2005). Upon reaching the plasma membrane, the N-terminal repeat region of aSyn binds to detergent-resistant membrane domains known as lipid rafts (Zabrocki et al., 2008). This process is vital, as any mutation in the N-terminus can prevent aSyn from binding to the plasma membrane, thereby inhibiting inclusion formation (Vamvaca et al., 2009). At non-toxic levels or in the absence of any toxic treatment, aSyn remains localized in the plasma membrane. However, our observation of large aSyn inclusions within the cytoplasm after 24 h of exposure to cymoxanil, which has an unknown mode of action, prompted us to investigate whether cymoxanil

directly induced the formation of aSyn inclusions or if it interfered with the delivery of aSyn to the membrane. This interference could occur through mechanisms such as the upregulation of aSyn expression or the inhibition of the secretory pathway. To verify this hypothesis, aSyn expression was induced in galactose-containing medium for 4 h (aSyn expression) and then halted by switching to a glucose-containing medium (aSyn repression). This transition was carried out immediately before exposure to 12.5 μg/mL of cymoxanil (Fig. 3B). As a control, cells were also maintained in galactose-containing medium during cymoxanil exposure. After 4 h, we observed the presence of small aSyn inclusions in both conditions. This observation suggests that cymoxanil indeed promoted the formation of aSyn inclusions of protein that had previously been localized in the plasma membrane.

3.4. Cymoxanil and metalaxyl induce formation of aSyn inclusions in a mammalian cell aggregation model of synucleinopathies

In order to validate the findings obtained in our yeast screen, we took advantage of a well-established model of aSyn inclusion formation based on the co-expression of aSyn and synphilin-1, an aSyn-interacting protein (Masaracchia et al., 2020; Dominguez-Meijide et al., 2021; Lázaro et al., 2014; Santos et al., 2022; McLean et al., 2001). In this model, we co-transfected human H4 cells with synphilin-1 and a modified C-terminal variant of wild-type aSyn named SynT (Masaracchia et al., 2020). Cells were then exposed to increasing concentrations of cymoxanil and metalaxyl (1, 10 and 20 μM) for 24 h. Afterwards, we assessed the

presence of aSyn inclusions through immunocytochemistry. In this analysis, at least 40 transfected cells were categorized into four groups based on the number of aSyn inclusions (Fig. 4A and B) or size (Fig. 4C). Our results showed that metalaxyl and cymoxanil affected aSyn inclusion formation with different trends. All concentrations of metalaxyl similarly decreased the percentage of cells lacking inclusions, and increased inclusion size. Cymoxanil clearly increased inclusion number and area depending on the dose, leading to a large increase in the percentage of cells with 1–4 inclusions, but not of cells with more than 4 inclusions. Furthermore, 20 μM of metalaxyl and, to a higher extent, cymoxanil decreased viability (Fig. 4D). We also found an increase in the percentage of cells displaying apoptotic nuclei with 10 μM of cymoxanil and 20 μM of metalaxyl (Fig. 4E and F).

4. Conclusions

Our study illustrates how the versatile humanized yeast model can provide significant insight into potential environmental factors associated with the development of synucleinopathies. Among the pesticides examined, metalaxyl and cymoxanil emerged as candidates. In the yeast model, cymoxanil triggered the formation of aSyn inclusions after 24 h of treatment, through a shift in the localization of aSyn from the plasma membrane to the cytosol. These findings suggest that cymoxanil has a direct impact on the cellular dynamics of aSyn, promoting its mislocalization and aggregation. To further underscore the significance of our observations, we sought validation in a mammalian aggregation model. Both metalaxyl and cymoxanil were found to induce aSyn inclusion formation, accompanied by a decrease in cell viability and an increase in apoptotic cells, highlighting the detrimental effects induced by the pesticides.

Collectively, our findings identified metalaxyl and cymoxanil as potential risk factors in the development of synucleinopathies, including PD. These results underscore the importance of further investigations into the relationship between pesticides and neurodegenerative diseases, providing valuable insights for future research and risk assessment in the context of synucleinopathies.

CRediT authorship contribution statement

Leslie Amaral: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Filipa Mendes:** Investigation, Formal analysis. **Manuela Côrte-Real:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **António Rego:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **Tiago F. Outeiro:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Susana R. Chaves:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143039.

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