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1 **Re-engineering of *CUP1* promoter and *Cup2/Ace1* transactivator to convert *Saccharomyces***
2 ***cerevisiae* into a whole-cell eukaryotic biosensor capable of detecting 10 nM of bioavailable**
3 **copper**

4
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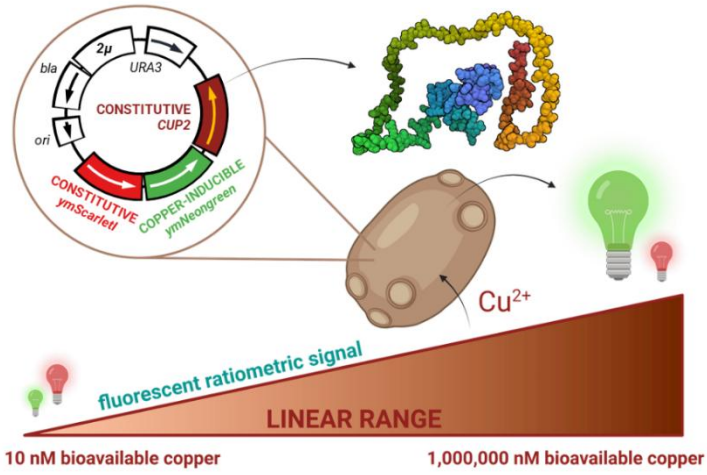
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23 1. Abstract

24 While copper is an essential micronutrient and a technologically indispensable heavy metal, it is toxic at high
25 concentrations, harming the environment and human health. Currently, copper is monitored with costly and low-
26 throughput analytical techniques that do not evaluate bioavailability, a crucial parameter which can be measured only
27 with living cells. We overcame these limitations by building upon yeast *S. cerevisiae*'s native copper response and
28 constructed a promising next-generation eukaryotic whole-cell copper biosensor. We combined a dual-reporter
29 fluorescent system with an engineered *CUP1* promoter and overexpressed Cup2 transactivator, constructing through
30 four iterations a total of 16 variants of the biosensor, with the best one exhibiting a linear range of 10^{-8} to 10^{-3} M of
31 bioavailable copper. The engineered variant distinguishes itself through superior specificity, detection limit, and
32 linear range, compared to other currently reported eukaryotic and prokaryotic whole-cell copper biosensors.
33 Moreover, the variant serves as a dual-sensing reporter for Cu^{2+} detection and cell viability, is relatively independent
34 of the cell's physiological status, disregards non-bioavailable copper and other heavy metals, and was validated on
35 real-world samples (contaminated with)/ containing interfering substances. Finally, by re-engineering the
36 transactivator, we altered the system's sensitivity and growth rate while assessing the performance of Cup2 with
37 heterologous activation domains. Thus, in addition to presenting the next-generation whole-cell copper biosensor,
38 this work urges for an iterative design of eukaryotic biosensors and paves the way toward higher sensitivity through
39 transactivator engineering.

40 **Keywords:** whole-cell biosensor, copper biosensor, bioavailability, *Saccharomyces cerevisiae*, transactivator
41 engineering

42 Graphical abstract



43

44 2. Introduction

45 Copper is the 25th most abundant element in Earth's crust (Emsley, 2001) and one of the three most important
46 metals in modern society (Sverdrup et al., 2014). It continues to rise in popularity, with each electric car containing
47 over 90 kg of copper, and power plants based on renewable sources needing 4-12-fold more copper than those based
48 on fossil fuels. In 2016, 20.2 million tonnes of copper were produced, with a projected annual increase of 6%
49 (Pietrzyk and Tora, 2018). However, copper mining is resource-intensive and polluting, with 70.4 tonnes of water
50 needed to mine one tonne of copper (Northey et al., 2013; Wang et al., 2007).

51 Copper is also an essential micronutrient that is toxic in high concentrations (Valko et al., 2005; Valko et al., 2006).
52 While the lack of copper induces neurological and blood disorders (Madsen and Gitlin, 2007), long-term exposure to
53 high copper concentrations is hepatotoxic (Briffa et al., 2020). On the other hand, copper serves as a fungicide,
54 algacide, insecticide, and wood preservative (World Health Organization, 2004). Thus, while widely used, copper is
55 closely monitored in drinking water, where it must not rise above 2.0 mg/l (31.5 μ M), as water with more than 2.5
56 mg/l of copper tastes bitter and with more than 4.0 mg/l induces nausea and vomiting (World Health Organization,
57 2011). Analytical laboratories currently monitor copper using inductively coupled plasma mass spectrometry (ICP-
58 MS), optical emission spectroscopy (ICP-OES), and flame atomic absorption (FAA), thus detecting 0.02 μ g/l (0.3
59 nM) of copper (World Health Organization, 2011). However, these methods are time-consuming, low-throughput,
60 require highly trained staff, and cannot distinguish between total and bioavailable copper.

61 When assessing pollution, the priority is to quantify bioavailable copper, i.e., copper interacting with living systems
62 (Magrisso et al., 2008), as its amount can differ drastically from that of total copper (Aruoja et al., 2009; Maderova et
63 al., 2011). However, bioavailable copper can be measured only through living organisms, which motivated the
64 development of whole-cell copper biosensors (Bhalla et al., 2016).

65 Most of the current whole-cell copper biosensors were constructed in prokaryotic organisms (Bereza-Malcolm et al.,
66 2015), e.g., *Escherichia coli* (Martinez et al., 2019; Pang et al., 2020), *Pseudomonas* sp. (Li et al., 2014; Maderova et
67 al., 2011), and *Cupriavidus metallidurans* (Chen et al., 2017; Leth et al., 2002). However, eukaryotic systems, such
68 as those employing model yeast *Saccharomyces cerevisiae*, might be more relevant to estimate the impact on human
69 health (Walmsley and Keenan, 2000).

70 *S. cerevisiae* is a non-pathogenic, well-studied, and easy-to-genetically-modify single-cell eukaryote. It is highly
71 resistant to copper (Marsit and Dequin, 2015; Steenwyk and Rokas, 2017), whose high concentrations it survives by
72 inducing several genes, including *CUP1* (Shi et al., 2021), which encodes Cu-metallothionein, a well-studied 61-
73 amino-acid protein that binds a surplus of toxic copper and cadmium ions (Ecker et al., 1986).

74 As it is activated by only a few transcription factors, the promoter of the *CUP1* gene is considered a model promoter
75 (Ball et al., 2016; Shen et al., 2001). Moreover, it is bound by only two transactivators, Cup2 and Hsf1, thus
76 responding only to toxic copper concentrations, heat shock, and oxidative stress (Liu and Thiele, 1996; Tamai et al.,
77 1994). In an environment with too much copper, activation of the *CUP1* gene is mediated by Cup2/Ace1
78 transactivator. This 225-amino-acid protein detects toxic copper (Singh et al., 2021), activating the transcription of
79 the *CUP1* gene through the C-terminal activation domain after binding copper ions in its N-terminal DNA-binding
80 domain (Li and Fay, 2019; Turner et al., 1998). As such, this transactivator could be employed in biosensor
81 construction.

82 In this work, we developed a next-generation eukaryotic whole-cell copper biosensor based on fluorescence
83 monitoring. For this purpose, we designed 16 *S. cerevisiae* plasmids encoding combinations of (i) a dual-reporter
84 fluorescent system, carrying one copper-inducible reporter and another standardising, constitutively-expressed
85 reporter, (ii) four different copper-inducible promoters, and (iii) nine rationally engineered variants of Cup2
86 transactivator. By this approach, we obtained a strain that detected copper with a linear range from 10^{-8} to 10^{-3} M. By
87 comparing strain performances, we also show that straightforward engineering of transactivators allows construction
88 of highly sensitive eukaryotic whole-cell biosensors.

89

90 **3. Materials and Methods**

91 **3.1. Media and growth conditions**

92 *E. coli* was grown overnight at 37°C at either 200 rpm in liquid 2xYT media (16.0 g/l tryptone, 10.0 g/l yeast extract,
93 5.0 g/l NaCl) or on LB plates (10.0 g/l tryptone, 5.0 g/l yeast extract, 5.0 g/l NaCl, 15.0 g/l agar), supplemented with
94 100 µg/ml of ampicillin. *S. cerevisiae* was grown at 30°C/180 rpm in chemically defined media without uracil (6.70
95 g/l Difco Yeast nitrogen base without amino acids, 20.0 g/l glucose, 0.77 g/l MP Bio complete supplement mixture

96 without uracil, and 20.0 g/l agar for solid media). When measuring copper response, Difco's YNB was exchanged
97 with Formedium's YNB without amino acids and copper (-ura-Cu medium), to which copper was added in the
98 required concentration as CuSO₄ (Merck, Germany). Biosensor specificity was tested with CoSO₄, NiCl₂·6H₂O,
99 ZnCl₂, PdCl₂(NCCH₃)₂, Cd(ClO₄), AgNO₃, and AuCl (Merck, Germany). Biosensor robustness was tested with
100 commercially available pharmacological Cu²⁺ supplements Oligosol Manganese Copper (72.6 μg Cu²⁺ and 72.8 μg
101 Mn²⁺ per 2 ml) and Oligosol Copper Gold Silver (3150 μg Cu²⁺, 70 μg colloidal Au, 1068 μg Ag⁺ per 100 ml)
102 (Labcatal, Mougins, France), as well as with concentrated plant fertilizer (0,002 % Cu²⁺, Florendi Jardin SAS,
103 France, Engrais NPK 6.6.6).

104 **3.2. Plasmid construction**

105 Plasmids were constructed in NEB Stable *E. coli* using Q5 polymerase, PCR Cloning Kit, Q5 Mutagenesis Kit, and
106 HiFi Assembly (New England Biolabs, Evry, France), with the details of the constructions given in Supplementary
107 material 1. Plasmid DNA was isolated with NucleoSpin Plasmid Mini Kit and extracted from an agarose gel with
108 NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, Duren, Germany). All constructs were verified by
109 restriction digest and Sanger sequencing, the results of which agreed with computed restriction maps. Primer
110 synthesis and Sanger sequencing were outsourced to Eurofins Genomics (Konstanz, Germany).

111 **3.3. Yeast strain construction**

112 In this work, all yeast strains were constructed by transforming BY 4742 (Baker Brachmann et al., 1998) as in Gietz
113 and Schiestl (2007). For the flow cytometry analysis, we used strains with overexpression cassette integrated into the
114 chromosome XI at EasyClone locus XI-1 (Jessop-Fabre et al., 2016), at which the neighbouring regions do not
115 interfere with gene expression. This chromosomal insertion ensured that each cell carried only one copy of the
116 expression cassette, thus producing a uniform cell population that could not be obtained with 2μ plasmid due to its
117 varying copy number. Strains with chromosomal insertions were constructed by amplifying overexpression cassettes
118 from previously constructed plasmids with Phusion polymerase (New England Biolabs, Evry, France) and
119 oligonucleotides eo-chrXI-f and eo-chrXI-r (Supplementary material 1). BY 4742 was transformed with PCR
120 products and the correct integrations of the transforming DNA fragments, as well as the lack of 2μ plasmids, were
121 confirmed via colony PCR with OneTaq polymerase (New England Biolabs, Evry, France).

122 **3.4. Fluorescence microscopy**

123 Cells were grown overnight in a -ura medium without or with 100 μM CuSO_4 until they reached a cell density of 10^7
124 cells/ml. Next, cells were centrifuged and imaged on a ZEISS LSM 980 confocal microscope with Airyscan 2 (Carl
125 Zeiss, Germany) under a 40x oil objective, using laser wavelengths of 488 and 561 nm. Images were processed using
126 Zeiss Zen Blue software.

127 **3.5. Flow cytometry**

128 Cells were grown overnight in -ura-Cu medium until the cultures reached $5 \cdot 10^6$ cells/ml and were then supplemented
129 with 100 μM CuSO_4 . Every 30 min, 100 μl of the culture was mixed with 200 μl PBS buffer (pH 7.4), and 25,000
130 events were recorded on BD LSRFortessa X-20, using 488 nm laser, 530/30 nm emission filter, and 505 nm long-
131 pass dichroic mirror. Each experiment was performed in triplicate. Data were analysed in the R computing
132 environment (R Core Team, 2021) using openCyto (Finak et al., 2014) and ggcyto packages (Van et al., 2018). The
133 gate was computed automatically to enclose 95% of the total events on the logicle-transformed forward and side
134 scatter areas. The ymNeogreen was assessed on the flowJo biexponential scale.

135 **3.6. Spectrophotometry**

136 Cells were grown in standard -ura medium to stationary phase, incubated at 4°C for 24 h, washed in sterile deionised
137 H_2O , diluted tenfold in -ura-Cu medium supplemented with CuSO_4 , and transferred into black sterile Greiner 96-well
138 microplates with a flat bottom. Every well contained 110 μl of the mixture and was prepared in triplicate.
139 Microplates were parafilmed to prevent evaporation and shaken at $30^\circ\text{C}/180$ rpm/16 h after which the parafilm and
140 microplate lid were removed, and the microplate was incubated for 20 min at room temperature.

141 Next, the microplate was imaged in Clariostar Plus (BMG Labtech, Ortenberg, Germany) preheated to 30°C . The
142 plate was first shaken at 700 rpm/6 min and then imaged for eight cycles with five flashes per well and cycle, with
143 shaking at 700 rpm/30 s between two cycles, after which the average fluorescence value was calculated for each
144 well. The ymNeogreen was excited at 470/15 nm and measured at 517/20 nm, using a 492.2 nm dichroic filter. The
145 ymScarletI was excited at 548/15 nm and measured at 591/20 nm, using a 568.2 nm dichroic filter. Data were
146 exported to .xlsx format and processed in the R programming environment.

147

148 **3.7. Growth curves**

149 Cells were grown in standard -ura medium to stationary phase, incubated at 4°C for 24 h, washed in sterile deionised
150 H₂O, diluted tenfold in standard -ura medium and transferred into black sterile Greiner 96-well microplates with a
151 flat transparent bottom so that every well contained 200 µl of the mixture. The microplate, covered with a lid, was
152 put into Clariostar Plus preheated at 30°C and OD₆₀₀ was measured in each well every 5 min, for 16 h. Before each
153 measurement cycle, the microplate was shaken at 600 rpm/30 s. Every condition was prepared and measured in
154 triplicate. Data were exported to .xlsx format and processed in the R programming environment. The obtained results
155 were consistent with the rate of colony growth on solid plates.

156 **3.8. Statistical analysis**

157 Statistical analysis was performed in the R programming environment (Supplementary material 3). The coefficient of
158 determination (r^2) was calculated by fitting a linear model to points within the linear range of the biosensor. The
159 detection limit (LOD) was defined as the minimal Cu²⁺ concentration at which the ymNeogreen/ymScarletI ratio
160 was higher than that of cells grown in -ura-Cu medium + its 3 standard deviations.

161

162 **4. Results**

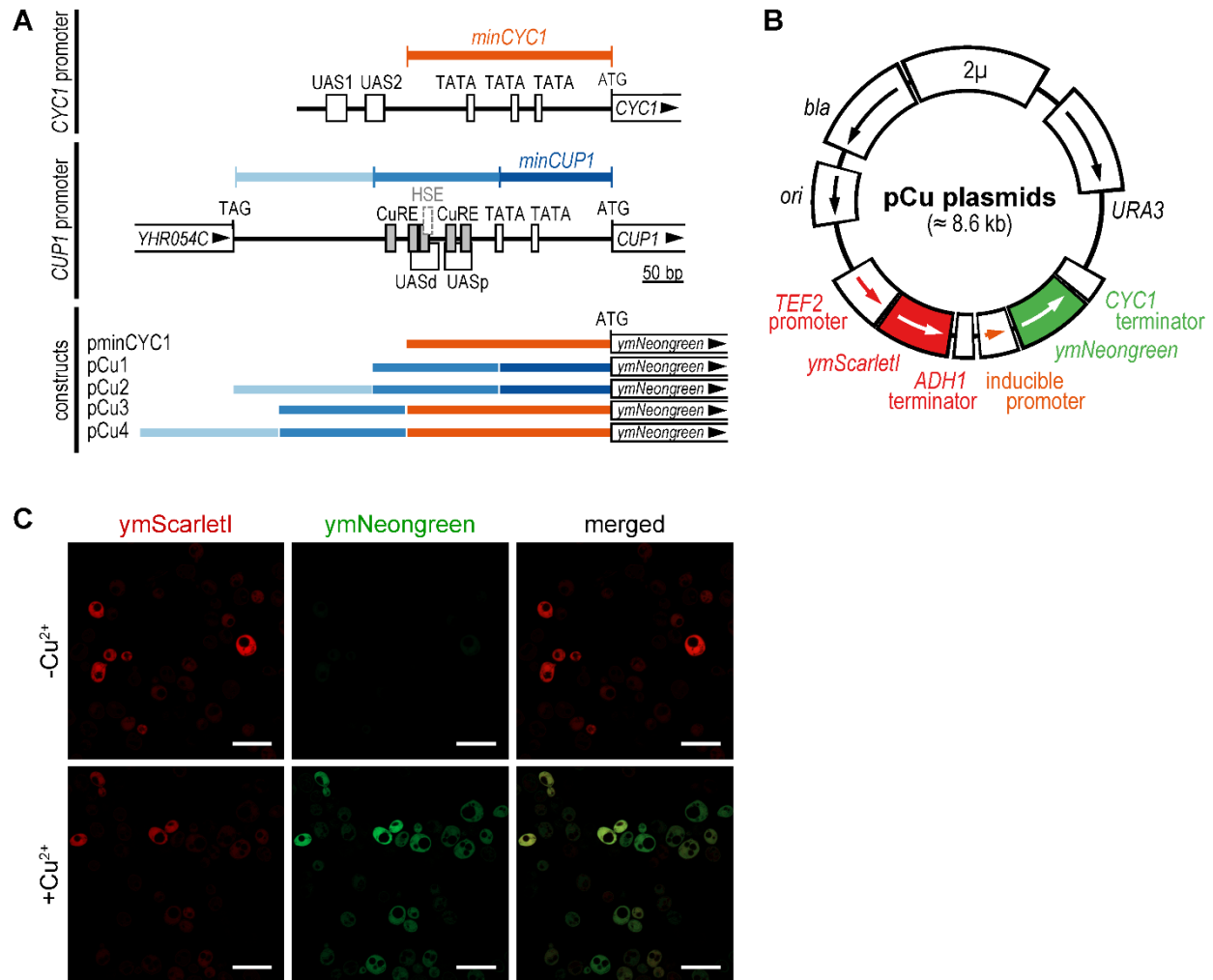
163 We developed a copper biosensor that leverages the native ability of yeast *S. cerevisiae* to detect and detoxify copper
164 ions. The engineered biosensor combines (i) a dual-reporter fluorescence-based system, with one reporter being
165 copper-inducible and another being constitutively expressed to accurately normalise the signal, (ii) modified *CUPI*
166 promoter and (iii) overexpressed Cup2 transactivator. The constructed biosensor is highly sensitive, detecting as little
167 as 10⁻⁸ M of bioavailable copper. While constructing it, we also re-engineered Cup2, truncating it and exchanging its
168 native activation domain with heterologous ones, thus highlighting the importance of balanced gene activation.

169 **4.1. The construction strategy**

170 To design an inducible reporter cassette, we first looked at the *CUPI* promoter, which is well-characterised and
171 induced above 10 µM Cu²⁺ (Ecker et al., 1986). Previous studies examined both the original *CUPI* promoter and a
172 hybrid *CUPI-CYCI* promoter (Thiele and Hamer, 1986), mainly focusing on the 150 bp regulatory region upstream
173 of the minimal *CUPI* promoter (Huibregtse et al., 1989) and disregarding an additional 170 bp of regulatory
174 sequence extending to the neighbouring ORF (Figure 1A). While the intensely studied region of 150 bp contains all

175 five Cup2 recognition elements, grouped into distal and proximal upstream activating sequences (UASd and UASp,
176 respectively), the overlooked region also holds putative recognition elements for transcription factors Yox1, Rap1,
177 Eds1, Rlm1, and Sum1 (Castro-Mondragon et al., 2022), which might affect induction. Moreover, the UASd also
178 encodes a heat shock element (HSE) responsive to heat and oxidative stress (Silar et al., 1991), thus promoting non-
179 copper mediated induction. We proceeded by inactivating the unwanted HSE as described previously (Tamai et al.,
180 1994) and combined both *CUP1* and *CYCI* minimal promoters (*minCUP1* and *minCYCI*, respectively) with *CUP1*
181 regulatory regions spanning either 150 bp or 320 bp, thus obtaining four versions of an inducible promoter (Cu1 to
182 Cu4).

183 After constructing inducible promoters, we placed them onto a 2 μ plasmid, where they drove the expression of
184 ymNeongreen (Figure 1B). The four constructed plasmids also encoded cassette for constitutive expression of
185 ymScarletI, driven by the *TEF2* promoter. Sequences of constructed plasmids were confirmed by restriction digest
186 and Sanger sequencing. Thus, while ymNeongreen measured Cu²⁺ concentration, ymScarletI reported on cell
187 density, with their ratio indicating Cu²⁺ concentration corrected by the number of cells. Both fluorescent proteins
188 were codon-usage optimised for yeast, displaying high *in vivo* brightness and photostability (Botman et al., 2019)
189 and fast maturation time (Bindels et al., 2017; Shaner et al., 2013). Finally, to ensure a robust signal even from non-
190 induced promoters, we selected *URA3* as a genetic marker, thus maintaining plasmid at over 20 copies per haploid
191 cell (Karim et al., 2013). Compared to cells not encoding any fluorescent proteins, strains encoding both
192 ymNeongreen and ymScarletI exhibited higher green and red fluorescence, even in the absence of copper. Moreover,
193 fluorescence micrographs showed that ymScarletI was indeed expressed in all conditions, whereas ymNeongreen
194 was significantly expressed only in the presence of Cu²⁺ (Figure 1C). High-contrast micrographs showed that every
195 cell expressing ymNeongreen also expressed ymScarletI. Conversely, in the presence of copper, each cell expressing
196 ymScarletI also expressed ymNeongreen. Thus, the system was fully functional.



197

198 **Figure 1:** Design of the four copper-inducible promoters. A) Structures of *CYC1* and *CUP1* promoters and four

199 copper-inducible promoters constructed in this work. The *CYC1* promoter has three TATA-boxes within the

200 sequence known as minimal *CYC1* promoter (*minCYC1*) and two upstream activating sequences (*UAS1* and *UAS2*)

201 outside it (Song et al., 2016). The *CUP1* promoter has two TATA-boxes and two upstream activating sequences

202 (*UASd* and *UASp*) that encode four Cup2 recognition elements (CuRE) and one heat shock element (HSE) (Thiele

203 and Hamer, 1986). Constructed plasmids carry minimal *CYC1* promoter (pminCYC1), truncated and full *CUP1*

204 promoter (pCu1 and pCu2, respectively) and two hybrid *CUP1-CYC1* promoters (pCu3 and pCu4). B) Plasmids

205 carrying copper biosensors encode β -lactamase (*bla*), pUC19 replication origin (*ori*), constitutive *TEF2* promoter and

206 *ADH1* terminator driving expression of yeast-optimised red fluorescent protein ymScarletI, copper-inducible

207 promoter and *CYC1* terminator driving the expression of yeast-optimised green fluorescent protein ymNeogreen,

208 yeast genetic marker (*URA3*), and replication origin of yeast plasmid 2 μ (2 μ). C) Fluorescence micrographs of cells

209 carrying pCu2 plasmid, grown in medium without copper (-Cu²⁺) or supplemented with 100 μM copper (+Cu²⁺),
210 expressing ymScarletI and ymNeogreen. Scale bar denotes 10.0 μm.

211

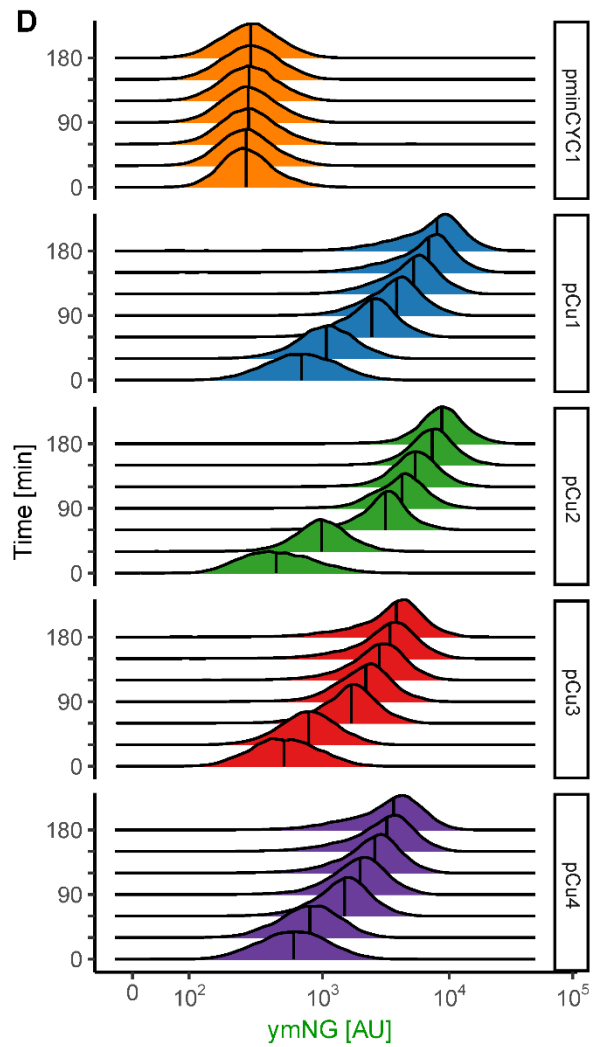
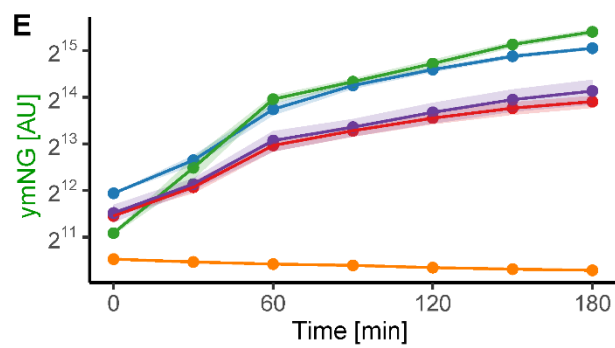
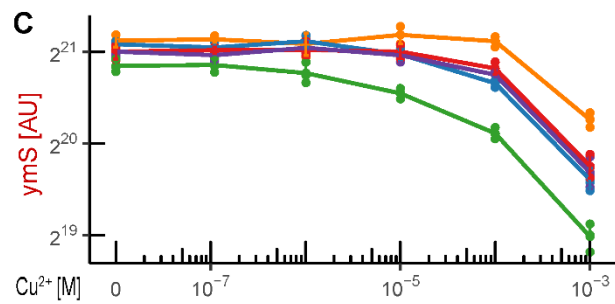
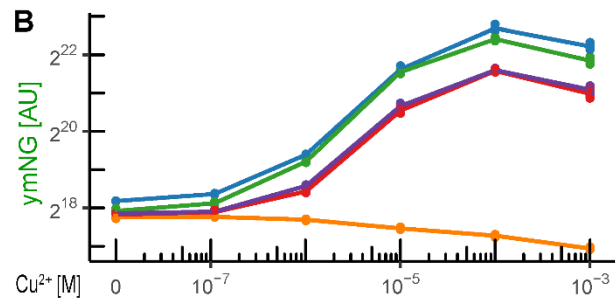
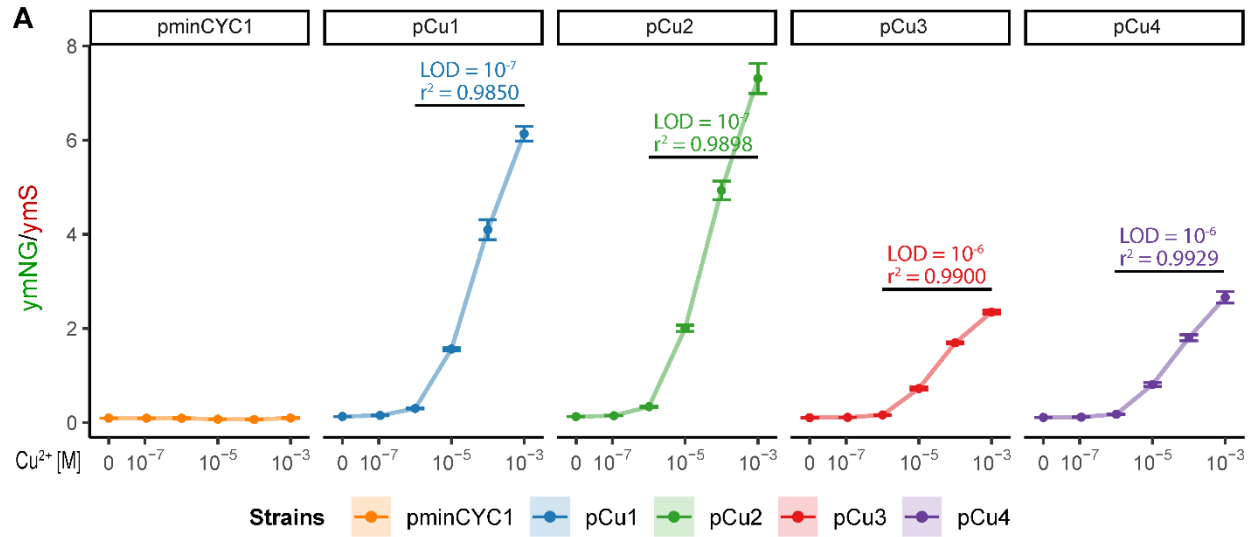
212 **4.2. Modifications of the *CUPI* promoter**

213 We then compared our four constructs carrying inducible promoters (pCu plasmids) with the construct carrying only
214 *minCYC1* promoter (pminCYC1). Cells carrying only pminCYC1 exhibited no change in their
215 ymNeogreen/ymScarletI ratio, whatever the Cu²⁺ concentration (Figure 2A). In contrast, cells carrying pCu
216 plasmids increased their fluorescence ratio as Cu²⁺ concentration rose, with their sensitivity and dynamic ratio
217 chiefly determined by their minimal promoter. The fluorescence ratio remained unchanged regardless of whether the
218 original inoculum was freshly grown or incubated for 14 days at 4°C, and regardless of whether cells were grown in
219 the copper-supplemented medium for 16 h or 24 h prior to measurement. However, after 24 h cells entered the
220 stationary phase, during which they started degrading fluorescent proteins. Thus, this biosensor requires a total
221 detection time of 16 h, as after sample preparation the cells need to exit the lag phase and complete three generations.
222 The constructs built upon *minCUP1* promoter (pCu1 and pCu2) outperformed those built upon *minCYC1* promoter
223 (pCu3 and pCu4), having a higher dynamic range and a detection limit reaching 10⁻⁷ M Cu²⁺.

224 A detailed study of the ymNeogreen and ymScarletI signals (Figures 2B and 2C) offered additional insights. The
225 changes in measured ratios originated only from ymNeogreen, except at the Cu²⁺ concentrations higher than 10⁻⁴ M,
226 at which ymNeogreen plateaued and ymScarletI decreased. Thus, measuring ymNeogreen and ymScarletI ratio at
227 high Cu²⁺ concentrations extended the biosensor's linear response to 10⁻³ M Cu²⁺. Interestingly, the declining
228 ymScarletI signal suggested a downregulation of the plasmid copy number, probably because the cells needed to
229 curb expression from the strongly induced promoter.

230 We also looked at the kinetics of the promoter induction, which also illuminated nuances of the *CUPI* regulation.
231 Flow cytometry showed symmetrical unimodal distribution for all four inducible promoters, with their median
232 fluorescence increasing over time (Figure 2D). Thus, for each strain, all cells within the population became induced.
233 The rate of promoter induction (Figure 2E) was similar among strains, except for pCu2, which carried the entire
234 *CUPI* intergenic region and had lower basal activity but reached pCu1 within 30 min. This observation suggests that
235 without Cu²⁺, the region proximal to *YHR054C* downregulates *CUPI*. Moreover, the *minCUP1* promoter was

236 required for this regulation, as the effect was absent in *minCYC1*-bearing pCu3 and pCu4. Thus, all four inducible
237 promoters were activated in the entire cell population within 30 minutes after adding Cu^{2+} , with the combination of
238 the *YHR054C*-adjacent region and *minCUP1* promoter fostering faster induction and higher dynamic range.



240 **Figure 2:** Copper biosensors with different promoters. A) The ymNeogreen/ymScarletI ratio of control construct
241 (pminCYC1) and engineered copper biosensors pCu1-pCu4. LOD = limit of detection, r^2 = coefficient of
242 determination. Black horizontal lines indicate a linear range. Error bars denote standard deviations (SD). B)
243 ymNeogreen signal. Error bars denote SD, while each point shows one measured sample. C) ymScarletI signal. D)
244 The distribution of ymNeogreen signal in cells induced with 100 μM Cu^{2+} , measured by flow cytometry. The
245 ymNeogreen cassette is integrated into the chromosome. Vertical lines within distributions denote median
246 fluorescence. E) Kinetics of promoter induction with 100 μM Cu^{2+} in cells with ymNeogreen cassette integrated
247 into the chromosome, measured by flow cytometry.

248

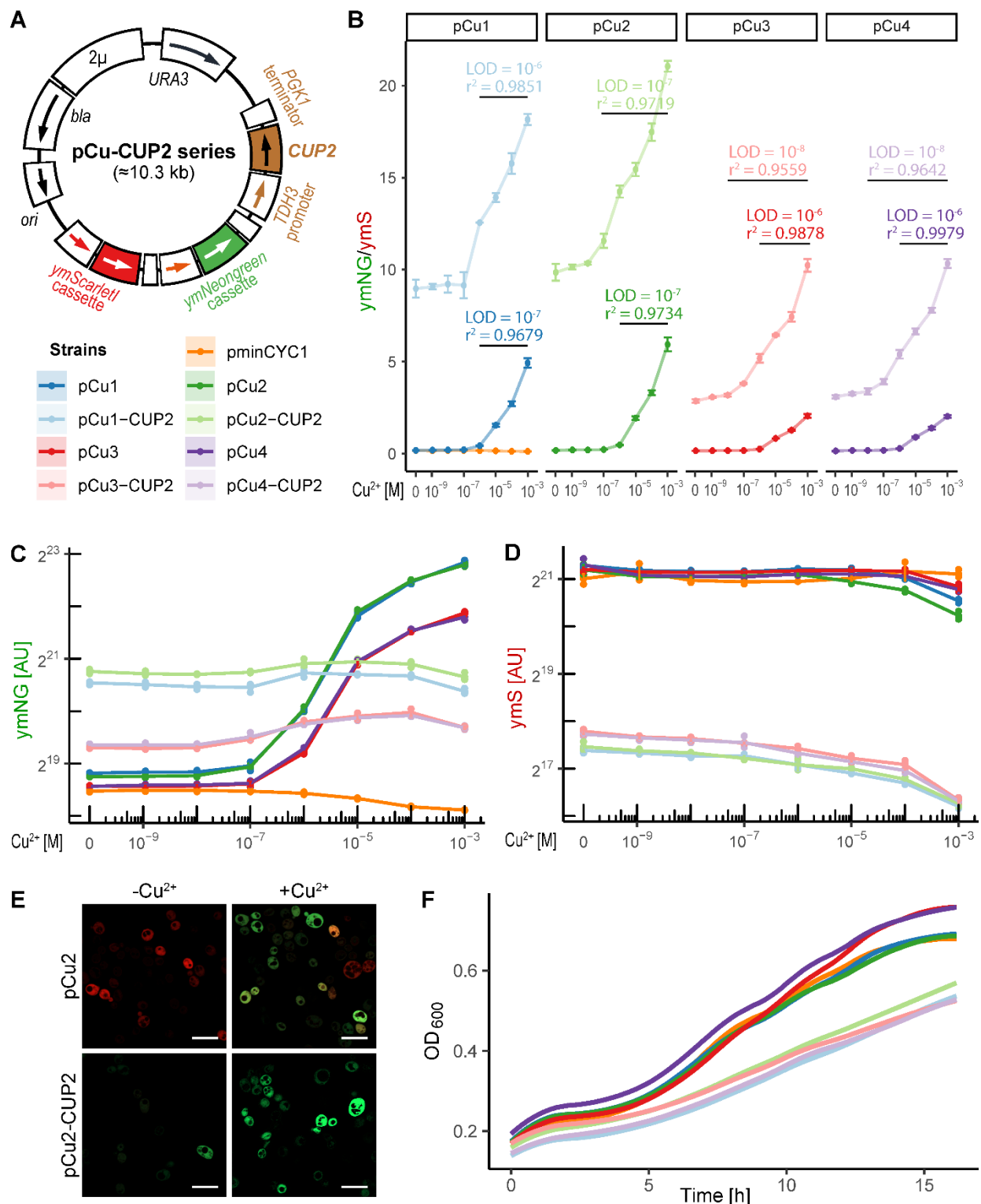
249 **4.3. Overexpression of Cup2 transactivator**

250 To increase the system's sensitivity, we added to pCu plasmids a strong constitutive *CUP2* expression cassette
251 driven by the *TDH3* promoter, thus obtaining the pCu-CUP2 plasmid series (Figure 3A). Compared to the pCu
252 series, all new constructs had a higher basal ymNeogreen/ymScarletI ratio, and several had better detection limits
253 and linear range (Figure 3B). While the detection limit of pCu1-CUP2 decreased, the detection limit of pCu2-CUP2
254 remained unchanged, although its linear range increased. The detection limit of pCu3-CUP2 and pCu4-CUP2 rose a
255 hundred-fold, as did their linear range, allowing them to detect as little as 10^{-8} M Cu^{2+} . Moreover, *minCYC1*-based
256 pCu3-CUP2 and pCu4-CUP2 outperformed *minCUP1*-based constructs in dynamic range and reproducibility. Thus,
257 plasmids pCu3-CUP2 and pCu4-CUP2 allowed for highly sensitive and reproducible detection of copper, ranging
258 from 10^{-8} to 10^{-3} M Cu^{2+} .

259 Interestingly, the higher baseline of pCu-CUP2 plasmids did not derive from ymNeogreen, whose expression rose
260 only two- to four-fold (Figure 3C). Instead, the effect was mainly due to a ten-fold drop in ymScarletI (Figure 3D),
261 which was also seen in fluorescence micrographs (Figure 3E). The rise of ymNeogreen and the drop of ymScarletI
262 were the strongest in the *minCUP1*-based pCu1-CUP2 and pCu2-CUP2, suggesting synergy between *minCUP1*
263 promoter and Cup2 binding sites.

264 The increased detection limit and linear range of pCu3-CUP2 and pCu4-CUP2 stemmed from the sensitivity and
265 accuracy of the ratiometric measurement as in these systems the rising Cu^{2+} concentration barely increased the
266 ymNeogreen signal, while the already faint ymScarletI signal decreased. Thus, although pCu3-CUP2 and pCu4-

267 CUP2 performed outstandingly, they never obtained the fluorescent intensity of pCu3 and pCu4. Finally, all pCu-
268 CUP2 plasmids slowed cell growth (Figure 3F), with cells retaining normal morphology (Figure 3E) but reaching
269 lower OD₆₀₀ values in the stationary phase (data not shown). Thus, pCu3-CUP2 and pCu4-CUP2 can detect copper
270 concentrations as low as 10⁻⁸ M but interfere with yeast growth, and so pCu1 and pCu2 might be more convenient
271 when such sensitivity is not required.



272

273 **Figure 3:** Copper biosensors overexpressing Cup2. A) Plasmids of the pCu-CUP2 series. The labels correspond to
 274 those in Figure 1B. B) Comparison of the ymNeogreen/ymScarletI ratio of copper biosensors with Cup2

275 overexpression (lighter colours) and without it (darker colours). C) ymNeongreen signal. D) ymScarletI signal. Other
276 labels are as in Figure 2. E) Fluorescence micrographs of cells carrying pCu2 and pCu2-CUP2 plasmids, grown in
277 medium without copper (-Cu²⁺) or supplemented with 100 μM copper (+Cu²⁺), expressing ymScarletI and
278 ymNeongreen. Scale bar denotes 10.0 μm. F) Growth curves of copper biosensors.

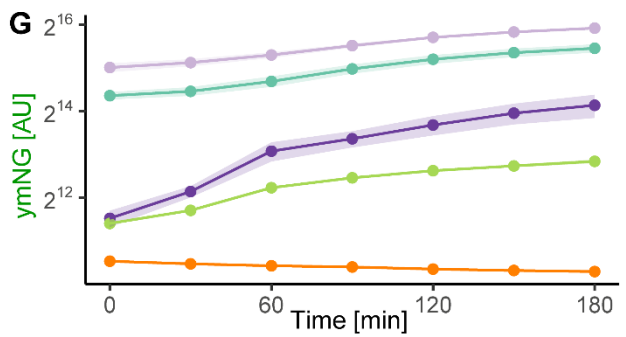
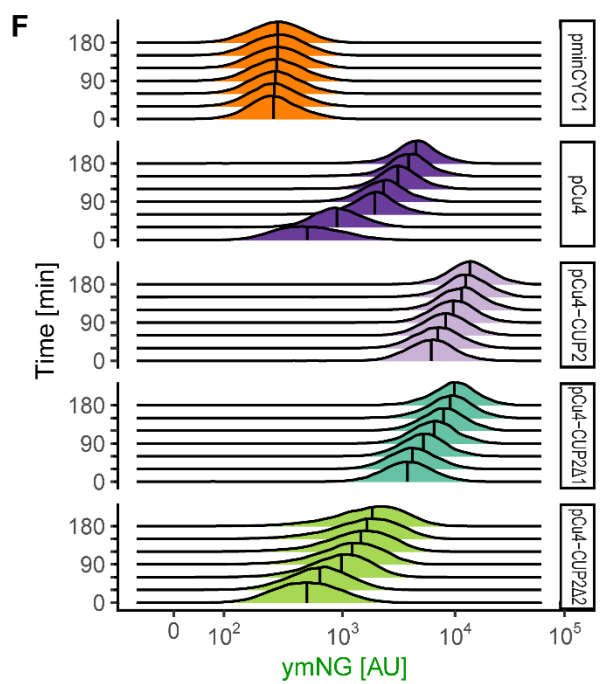
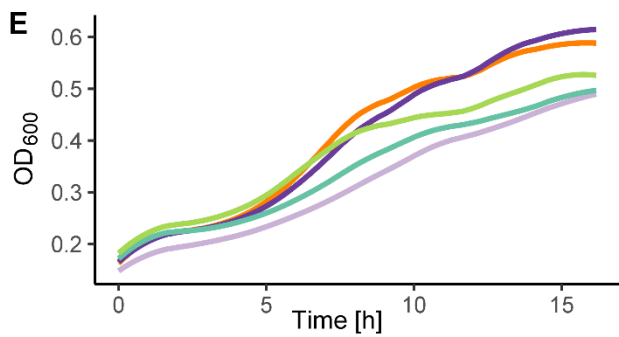
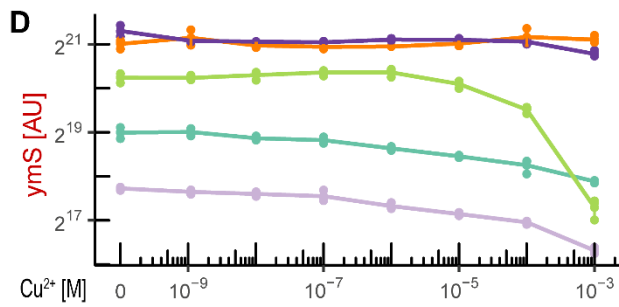
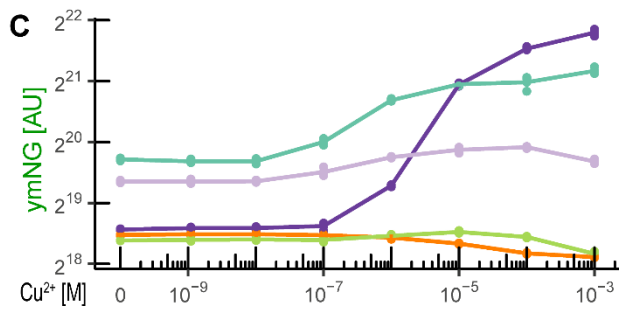
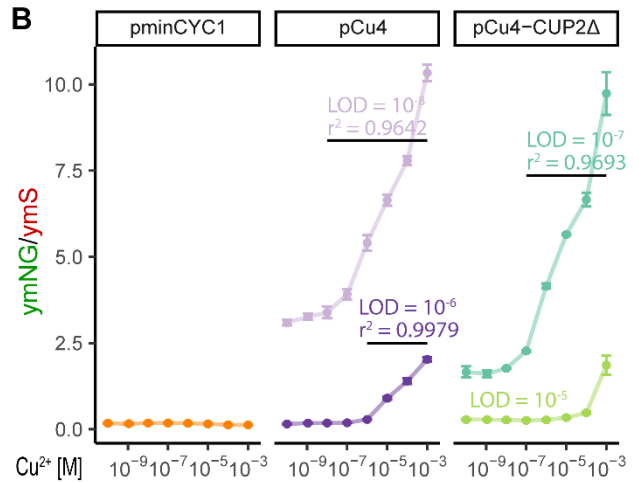
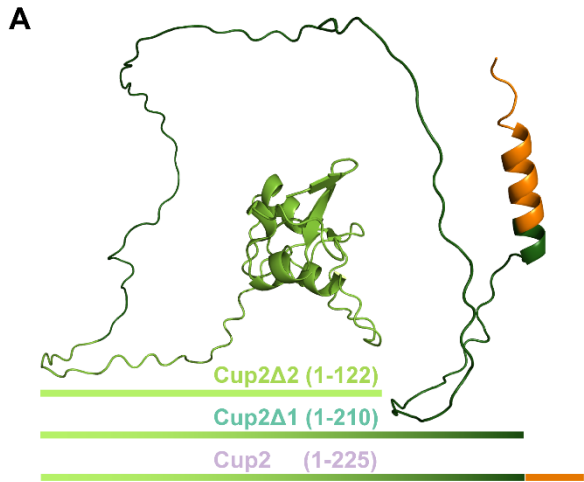
279

280 **4.4. Partial deletions of Cup2 transactivator**

281 We wondered if we could fine-tune the system further, boosting the growth and fluorescent signal of pCu4-CUP2
282 cells while retaining their extreme Cu²⁺ sensitivity. As overexpressed transactivators can slow growth through overly
283 strong activation domains (Gill and Ptashne, 1988; Meyer et al., 1989), we tried to pinpoint the Cup2 activation
284 domain. Alphafold's prediction of Cup2 structure (Figure 4A, Uniprot Accession P15315) showed that the
285 transactivator's DNA-binding and regulatory regions, residing in the N-terminal half of the protein, were well-
286 structured. On the other hand, the C-terminal half of the protein appeared intrinsically disordered (Ruff and Pappu,
287 2021), which is characteristic of activation domains (Ravarani et al., 2018). Moreover, a localised search for
288 activating sequences identified a 75% match to the 9aaTAD pattern (Piskacek et al., 2016) in the Cup2 211-219
289 residues that fold into solitary alpha-helix at protein's C-terminus.

290 Based on structural information, we tried to dampen the activity of plasmid-based Cup2. Thus, we constructed two
291 truncated Cup2 variants, lacking 15 or 103 C-terminal amino acid residues (Cup2Δ1 and Cup2Δ2, respectively;
292 Figure 4A). The constructs' properties differed markedly. Compared to pCu4-CUP2, plasmid pCu4-CUP2Δ1
293 imparted poorer detection limit and linear range but increased dynamic range (Figure 4B) and strengthened
294 ymNeongreen and ymScarletI signals (Figures 4C and 4D). On the other hand, plasmid pCu4-CUP2Δ2 effectively
295 repressed the promoter, lowering its detection limit and ymNeongreen signal below those of pCu4 (Figures 4B and
296 4C), thus suggesting that Cup2Δ2 competitively inhibits DNA binding of the endogenous, chromosomally encoded
297 Cup2. Such inhibition also interfered with regular copper-detoxifying Cup1 response, thus undermining growth at
298 higher copper concentrations (Figure 4D). Interestingly, although pCu4-CUP2, pCu4-CUP2Δ1, and pCu4-CUP2Δ2
299 carried the same promoters, they differed in ymScarletI signals, suggesting that plasmid copy number also depended
300 on the protein that plasmid expressed. Finally, compared to pCu4-CUP2, plasmid pCu4-CUP2Δ1 had no impact on
301 the rate of growth (Figure 4E) and promoter induction (Figures 4F and 4G), while pCu4-CUP2Δ2 grew faster than

302 pCu4-CUP2 but slower than pCu4 (Figure 4E). As such, growth curves also imply that cells grew slowly due to
303 overly strong activation domains. Overall, truncated Cup2 variants performed worse than full-length Cup2,
304 suggesting that potent activation domains slow growth and weaken the fluorescent signal but significantly increase
305 detection limit and linear range.



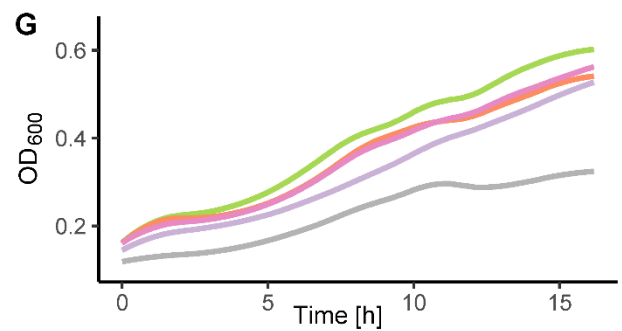
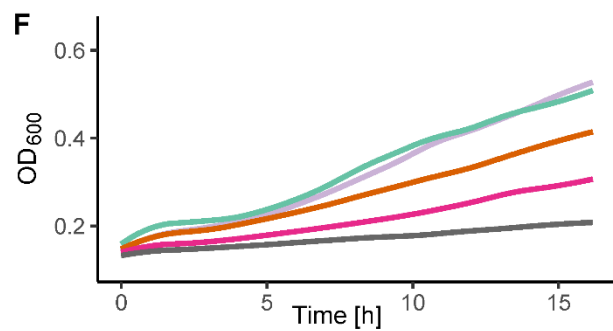
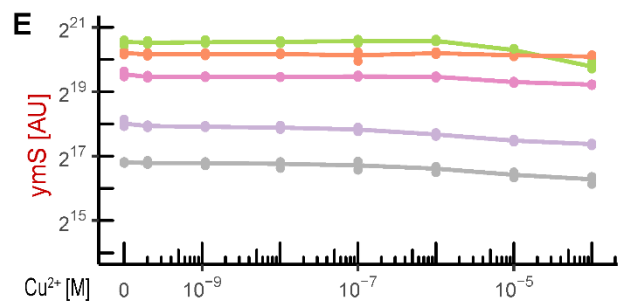
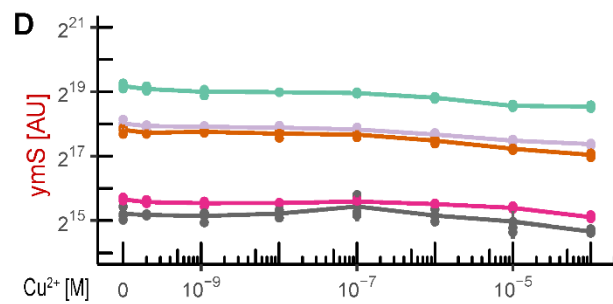
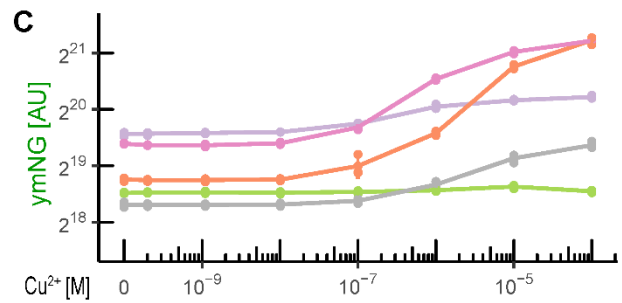
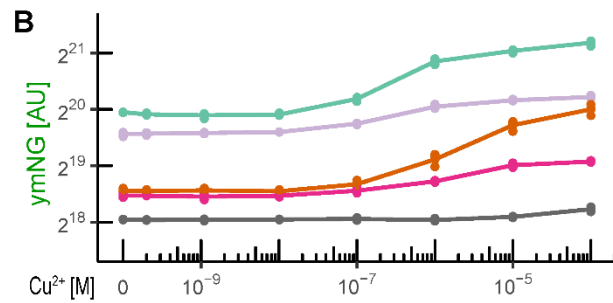
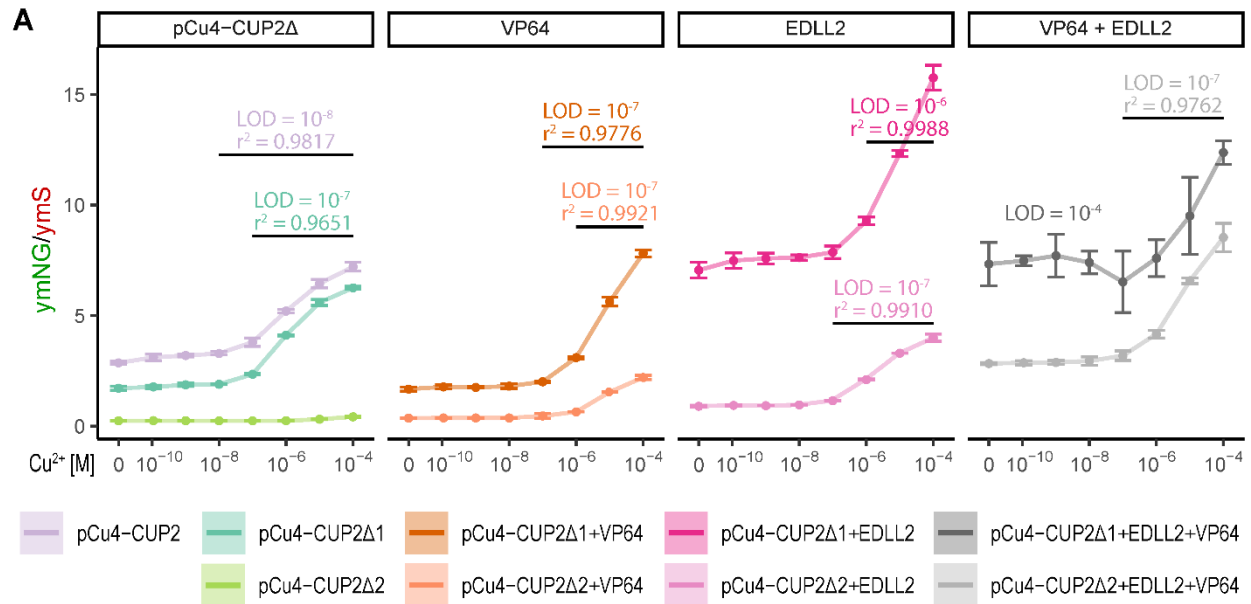
307 **Figure 4:** Copper biosensors overexpressing truncated Cup2. A) Alphafold-predicted structure of Cup2 and its
308 trimmed variants. Cup2 Δ 1 contains both light-green and dark-green regions of Cup2, but not the orange region,
309 while Cup2 Δ 2 contains only the light-green region. Numbers in parentheses denote amino acid residues contained
310 within each variant. B) The ymNeongreen/ymScarletI ratio for control construct, pCu4 biosensor without and with
311 Cup2 overexpression, as well as biosensors overexpressing trimmed Cup2 variants. C) ymNeongreen signal. D)
312 ymScarletI signal. Other labels are as in Figure 2. E) Growth curves. F) The distribution of ymNeongreen signal in
313 cells induced with 100 μ M Cu²⁺, measured by flow cytometry. The ymNeongreen cassette is integrated into the
314 chromosome. Vertical lines within distributions denote median fluorescence. G) Kinetics of promoter induction with
315 100 μ M Cu²⁺ in cells with ymNeongreen cassette integrated into the chromosome, as measured by flow cytometry.

316

317 **4.5. Cup2 variants with heterologous activation domains**

318 As previous results suggested that a more potent activation domain benefits the biosensor, we fused truncated Cup2
319 variants with strong heterologous activation domains. Thus, to both Cup2 Δ 1 and Cup2 Δ 2, we appended activation
320 domains VP64 from herpes simplex virus (Beerli et al., 1998), EDLL2 from *Arabidopsis thaliana* (Naseri et al.,
321 2017), or their combination.

322 Hybrid Cup2 transactivators underlined the importance of balanced activation. Fusing Cup2 Δ 1 with either VP64 or
323 EDLL2 lowered the systems' detection limit ten-fold, and linking both domains to it lowered it even more (Figure
324 5A). On the contrary, a fusion of Cup2 Δ 2 with either domain was beneficial, with the construct carrying both
325 activation domains together rivalling the non-truncated Cup2. The ymNeongreen and ymScarletI also reflected
326 variation in construct performance (Figures 5B, 5C, and 5D, 5E, respectively), with the expression of ymScarletI
327 closely matching the growth rates (Figures 5F and 5G). Thus, adding domains to mostly intact Cup2 creates an
328 overpowering transactivator, harming the system. In contrast, adding the same domains to the solitary DNA-binding
329 domain of Cup2 is beneficial.



331 **Figure 5:** Copper biosensors overexpressing Cup2 with different activation domains. A) Comparison of the
332 ymNeongreen/ymScarletI ratio for pCu4 biosensor overexpressing Cup2, its trimmed variants, and combination of
333 trimmed variants with VP64 and EDLL2 activation domains. B) ymNeongreen signal of Cup2Δ1-based variants. C)
334 ymNeongreen signal of Cup2Δ2-based variants. D) ymScarletI signal of Cup2Δ1-based variants. E) ymScarletI
335 signal of Cup2Δ2-based variants. F) Growth curves of Cup2Δ1-based variants. G) Growth curves of Cup2Δ2-based
336 variants.

337

338 **4.6. Biosensor specificity**

339 As Cup2 binds Ag^+ *in vitro* (Buchman et al., 1989), we determined the specificity of our system by trying to
340 detect related metals: Co^{2+} , Ni^{2+} , Zn^{2+} , Pd^{2+} , Cd^{2+} , Ag^+ , or Au^+ in concentrations up to 10^{-3} M (Supplementary
341 material 2). Higher concentrations of cationic metals negatively affected yeast cells, decreasing their
342 ymNeongreen/ymScarletI ratio in most conditions, but no significant response was observed for any of the
343 related metals. Thus, our system is highly specific for Cu^{2+} ions.

344

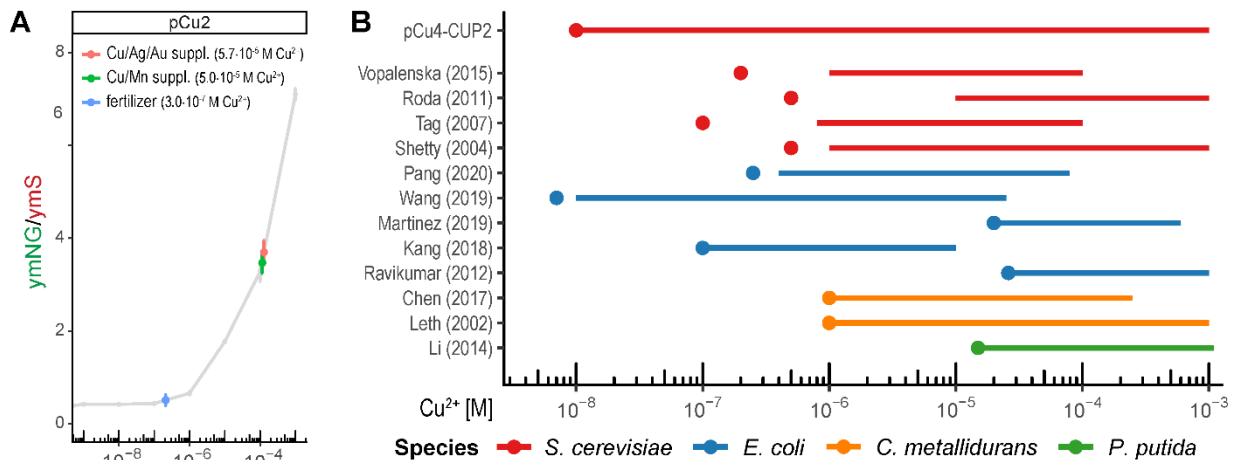
345 **4.7. Biosensor robustness**

346 To check for the robustness of the biosensors pCu2 and pCu4-CUP2, we measured bioavailable copper in first
347 decimal dilutions of commercially available pharmacological Cu^{2+} supplements containing a mixture of copper and
348 manganese (Cu/Mn), or copper, silver, and gold (Cu/Ag/Au). Biosensor pCu2 detected $1.15 \cdot 10^{-4}$ M Cu^{2+} in Cu/Mn
349 supplement (advertised value: $0.5 \cdot 10^{-4}$ M) and $1.28 \cdot 10^{-4}$ M Cu^{2+} in Cu/Ag/Au supplement (advertised value: $0.57 \cdot 10^{-4}$
350 M) (Figure 6), although its growth was inhibited in the presence of toxic Ag^+ ions. The pCu2 also detected $2.12 \cdot 10^{-7}$
351 M Cu^{2+} in the third decimal dilution of a concentrate of a commercial plant fertilizer (advertised value: $3 \cdot 10^{-7}$ M).
352 Higher fertilizer concentrations did not produce a stronger signal, suggesting limited copper bioavailability. The
353 pCu4-CUP2 detected $4.64 \cdot 10^{-5}$ M Cu^{2+} in Cu/Mn supplement (advertised value: $5.70 \cdot 10^{-5}$ M), but it
354 produced/exhibited excessive variations when incubated with Cu/Ag/Au supplement or plant fertilizer, probably as
355 overexpressed *CUP2* made the strain more susceptible to growth inhibitors. Thus, both pCu2 and pCu4-CUP2
356 detected bioavailable copper in commercially available samples, and pCu2 was more robust with relevant values.

357

358 4.8. Comparison with other whole-cell copper biosensors

359 Finally, we compared pCu4-CUP2, our best performing biosensor, with twelve others reported in the literature
360 (Figure 6B). This comparison showed that pCu4-CUP2 outperformed other eukaryotic whole-cell biosensors, having
361 at least a hundred-fold wider linear range and at least a ten-fold better detection limit. While the state-of-the-art
362 prokaryotic *E. coli*-based biosensors performed better than eukaryotic ones, only one had a detection limit
363 comparable to pCu4-CUP2 and none had as wide linear range. Thus, the system developed in this work distinguishes
364 itself by an outstanding combination of specificity, detection limit, and linear range.



365

366 **Figure 6:** Robustness of the developed biosensor and comparison to other published copper biosensors. A)
367 Performance of the pCu2 biosensor in samples containing commercial Cu²⁺ supplements, with the grey line denoting
368 biosensor calibration curve and the coloured labels showing biosensor response to each commercial sample. Values
369 in the parentheses indicate Cu²⁺ concentration as advertised by the manufacturer. B) Comparison of the pCu4-CUP2
370 with other published copper biosensors. The horizontal line marks the linear range and the point next to it marks the
371 detection limit, with colours denoting different species.

372 5. Discussion

373 In this work, we redesigned yeast *S. cerevisiae* into a whole-eukaryotic-cell dual-reporter biosensor able to detect 10⁸
374 to 10⁻³ M of bioavailable copper. For this purpose, we constructed two native and two hybrid variants of
375 the *CUP1* promoter and tested them in wild-type cells and in cells overexpressing Cup2 transactivator. While the
376 native promoter variants performed better in wild-type cells, they were surpassed by hybrid promoter variants in cells
377 overexpressing Cup2 transactivator. However, the abundance of Cup2 slowed down cell growth, prompting us to
378 engineer its activation domain. While trimming this domain helped growth but worsened sensitivity, strengthening it
379 worsened both metrics, suggesting further development should rely on subtler domain modifications.

380 Biosensors developed in this work can detect a concentration of bioavailable copper as low as 10 nM and have
381 extremely wide linear ranges, from 0.01 μ M to 1000 μ M (5 log scale), thus ranking amongst the most sensitive
382 whole-cell copper biosensors. Moreover, they measure only copper capable of entering the eukaryotic cell,
383 disregarding non-bioavailable copper and other heavy metals, and were validated on real samples, containing
384 interfering substances. Most other whole-cell biosensors are prokaryotic, which are less sensitive and have a
385 narrower linear range. However, they still perform better than existing eukaryotic systems. Thus, the system
386 described in this work distinguishes itself through high specificity, low detection limit, and wide linear range.

387 This work also relies on the design of a novel ratiometric, i.e., dual-reporter whole-cell biosensor, and is one of the
388 only few such systems currently described (Mirasoli et al., 2002; Roda et al., 2011). The ymScarletI, as an internal
389 reference system, monitored viability, allowing us to evade OD₆₀₀ measurement, an unreliable score of cell density in
390 polluted and toxic environments. Thus, the presented ratiometric system has clear advantages over single reporters,
391 extending the biosensor's detection limit and linear range, and serving as a dual-sensing reporter for Cu²⁺ detection
392 and cell viability.

393 We constructed and compared four versions of the *CUP1* promoter without heat shock element (HSE), thus making
394 it more independent of the cell's physiological status. We showed that the *YHR054C*-adjacent sequence cooperates
395 with the *minCUP1* promoter to affect induction kinetics and that the *minCUP1* promoter considerably affects the
396 biosensor's detection limit and dynamic range, although it does not carry Cup2-binding sites. Interestingly, Badi and
397 Barberis (2002) noticed that the region upstream of *CUP1* UAS restores impaired activation of *CUP1* transcription,
398 even suggesting that the *YHR054C* sequence itself has a regulatory role. Moreover, overexpression of Cup2 benefited

399 the *minCYC1*-based biosensors but not the *minCUP1*-based biosensors, suggesting an interaction between the Cup2-
400 binding region and *minCUP1* promoter, which is disrupted in the hybrid promoter but restored at higher Cup2
401 concentration. Thus, the *CUP1* promoter seems to have evolved as one compact, well-tuned transcription unit, with
402 both Cup2-binding and non-binding segments working together to regulate transcription.

403 The Cup2 overexpression enhanced otherwise under-performing *minCYC1*-based constructs. This result highlighted
404 difficulties in predicting construct performance and underlined the importance of testing many variants. However,
405 overexpression of Cup2 lowered ymNeongreen and ymScarletI signals, with the overexpression system ultimately
406 performing well because of the dual-reporter ratiometric approach.

407 The Cup2 overexpression also hampered growth, probably through squelching (Schmidt et al., 2016), i.e., cell-wide
408 disturbance of transcription caused when overexpressed transactivator binds up too much Mediator, causing its cell-
409 wide shortage. In support of this hypothesis, Sanborn et al. (2021) recently demonstrated that the Cup2 activation
410 domain directly binds Mediator. We restored growth by trimming the C-terminal half of Cup2 but thus created a
411 transcriptional repressor that competes with chromosomally encoded Cup2 for binding sites, blocking copper
412 response. These results suggest that similar overexpression of the DNA-binding domain alone could serve as a
413 general strategy for damping unwanted transcriptional responses.

414 We further adjusted the biosensor by building upon or exchanging native Cup2 activation domain with foreign
415 sequences, e.g., viral VP64 or plant EDLL2 domains. We noted that strong activation domains slowed growth,
416 especially when present together, paralleling the observation that domains present *in tandem* bind Mediator stronger
417 than expected due to the synergistic effect of their interaction sites (Sanborn et al., 2021). Curiously, this effect could
418 be employed to limit the growth rate, and thus harnessed for biocontainment and biosafety (Wang and Zhang, 2019).

419 We constructed and compared a total of 16 copper biosensors, which is an unusual approach given that most
420 publications characterise only one variant. However, such an approach allowed us to deduce some design guidelines
421 and inspired further modifications. For example, while we focused on exchanging entire activation domains, an
422 alternative approach would be to change single amino acids (Sanborn et al., 2021), to allow for more precise tuning
423 of the signal. Moreover, the detection limit might be improved by deleting an array of *CUP1* genes, which down-
424 regulate their own expression (Wright et al., 1988) and are over-induced in strains overexpressing Cup2 (Huibregtse

425 et al., 1989). Additional strategies could rely on modifying chromosomally encoded *CUP2* or introducing more Cup2
426 binding elements into the inducible promoter.

427

428 **6. Conclusion**

429 In this work, we leveraged the native copper response of yeast *S. cerevisiae* and redesigned it. We constructed the
430 next generation eukaryotic whole-cell copper biosensor exhibiting very high sensitivity, specificity, and wide linear
431 range, allowing detection of bioavailable copper from 10^{-8} to 10^{-3} M. For this purpose, we combined an
432 unprecedented dual-reporter strategy with significant promoter and transactivator engineering. While the system
433 performs admirably, its signal is intended to be measured only after overnight incubation under standard growth
434 conditions, in the medium supplemented with the analyte. In our upcoming work, we plan to further fine-tune
435 fluorescent signal by introducing point mutations in activation domains and to construct an analogous ratiometric
436 system for the detection of antibiotics. As such, our approach points out a new paradigm for developing and
437 improving any eukaryotic whole-cell biosensor.

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442 BioRender.com.

443 **8. Supplementary materials**

444 Supplementary material 1: Details of the plasmid and strain construction. Supplementary material 2: Biosensor
445 response to copper-related cationic metals. Supplementary material 3: List of mean values and corresponding
446 standard deviations depicted in Figures 2-5.

447 **9. Competing Interests**

448 The authors declare no competing interests associated with the manuscript.

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452 **11. CRediT author statement**

453 **Bojan Žunar:** Conceptualization, Methodology, Software, Validation, Investigation, Formal analysis, Visualization,
454 Writing - Original Draft, Writing - Review & Editing. **Christine Mosrin:** Resources, Writing - Review & Editing.
455 **Hélène Bénédicti:** Resources, Supervision, Writing - Review & Editing. **Béatrice Vallée:** Conceptualization,
456 Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

457 **12. Literature**

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