I would like to thank again the reviewers and the editor for the positive assessment of my manuscript. Below I respond to the reviewer comments in detail and I indicate which changes have been made to the manuscript (major changes in blue). Figures 4, 6, SI7 and SI8 have also been modified, and a new figure (SI9) has been added.

I hope that my revised manuscript is now suitable for publication in PCI and I am looking forward to hearing from you.

Review by anonymous reviewer 1, 05 Jul 2024 08:23

I would like to thank the author for the changes made in manuscript, which is now clearer and sounder. Analyses are reproducible, all data and scripts are available on Github.

My remaining comments are minor:

- I am still doubful about the use of "(somehow overlooked)" phrase in the abstract. I believe "suggesting the importance" is well enough and less subjective.

The terms “(somehow overlooked)” have been removed.

- P2 l53: "we" --> "I" for consistency with the rest of the text?

Done.

- Fig4: consider adding in the graphical legend the signification of the node width (switch node score)

In the previous version, the node of Erythrose 4-phosphate was bigger to highlight its specific role. In the new version, the node width indicates switch score, and a legend has been added.

- Fig6: what is plotted is not clear: in x the dot is the average and the std is plotted as vertical bars?

The legend of the figure has been modified to clarify this point.

- In the discussion (L197 p10) could the discrepencies occur simply because FBA does not account for transcriptional regulation, therefore some switch metabolites could be false positives? Could constraining fluxes while taking additional data into account (transcriptomics), or maybe using alternatives to FBA (eg RBA? https://doi.org/10.1042/BST20160436) to compute the fluxes highlight more biologically relevant switches?

There are important differences between reporter metabolites and switching points, but we do not know the ground truth. As stated in lines 141-147, some reporter metabolites are questionable: they have no known link with the environmental conditions studied.

On the other hand, ISIS relies entirely on the quality of flux estimations. Every method leading to better estimations (e.g. using transcriptomic data, or considering enzyme constraints as in RBA) will definitely improve the identification of switch metabolites.

These points are now further discussed (l. 200-201 and 210-211).

Review by anonymous reviewer 2, 02 Jul 2024 10:02

The author has significantly improved the manuscript's flow and clarity by providing additional explanations, details, and comparisons to other existing methods in this area.

Regarding my previous remark about the possible bias in identifying switches due to variability in flux solutions, the newly introduced section, "Robustness to Flux Sampling," is a welcome addition and clearly demonstrates why alternative flux solutions should be analyzed. The author observed that multiple metabolites, not identified in the original study as switches, became switches when studying alternative solutions, and vice versa.

This observation raises questions about the reliability of identified metabolic switches in the studies preceding the introduced section. The author's statement on page 8, lines 169-170, "Exploring the flux space is therefore useful to confirm the identification of switch points, but it also leads to artificial switch points," is misleading. Exploring the flux space allows us to consider all possible outcomes given the experimentally observed data and network topology, whereas considering only one arbitrary flux solution provides just one snapshot. Therefore, the claim that sampling leads to "artificial switch points" is not necessarily accurate.

With flux sampling, several alternative pathways starting from the same substrate and arriving at the same product could appear, not related to the change of environmental conditions under investigation. This leads to switch points that are not relevant, as e.g. all the lipid intermediates that have appeared for *S. cerevisiae* under nitrogen limitation. Figure SI 9 has been added to illustrate with one example taken from this case study how irrelevant switch points could appear. Nevertheless, I agree that not all the new candidates are to be discarded. The manuscript has been modified to clarify this point (l. 166-173).

Similarly, on page 10, lines 201-202, the statement "false positives may be generated" follows the same reasoning. One may ask what the ground truth is on which the author bases their conclusion about the false positivity of these outcomes.

This part has also been modified, to highlight that some of these candidates could be false positives, and others not (l.205-208).

A common approach to addressing such bias in this line of research is to explore the flux solution space with a statistically significant number of samples, analyze the obtained distributions, and draw conclusions based on chosen statistical criteria. This method would allow the author to provide a probability measure of identified switches. I recommend such an approach for the presented studies, as the current conclusions may be drawn from a flux solution that is an outlier (or on the tails) in the distribution of flux solutions.

I agree that my method rely on the quality of the flux solution. As discussed in l. 208-213, we could expect that these estimations become more and more reliable.

Using ISIS repeatedly with flux sampling is a first approach to test the robustness of the results, which presents however some limitations (as discussed above). Using flux distribution is appealing, but I do not see how to adapt my method to use directly flux distributions. This should deserve future works.

Additionally, the number of samples (1000) in the provided robustness sampling study is insufficient for statistical significance in the case of large-scale metabolic networks.

The number of samples has been increased to 10^4. Figure 6, SI7 and SI8 have been changed, but the results and the conclusion remain the same.

Minor Corrections:

Page 4, line 108: “shows” should be changed to “show.”

Done.

Page 8, line 159: “vey” should be corrected to “very.”

Done.